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Effect of Pentacyclic Triterpenes Found in *Perilla frutescens* Alone or in Combination with Resveratrol on Skin Tumor Promotion by 12-*O*-tetra-decanoylphorbol-13-acetate

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By

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Dedication

This dissertation is dedicated to my lovely father, mother, and two sisters.

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Effect of Pentacyclic Triterpenes Found in *Perilla frutescens* Alone or in Combination with Resveratrol on Skin Tumor Promotion by 12-*O*-tetra-decanoylphorbol-13-acetate

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A series of pentacyclic triterpenes found in *P. frutescens*, including ursolic acid (UA), oleanolic acid (OA), augustic acid (AA), corosolic acid (CA), 3-epi-corosolic acid (3-epiCA), maslinic acid (MA), and 3-epi-maslinic acid (3-epiMA) were evaluated for their effects on skin tumor promotion by 12-O-tetradecanoylphorbol-13-acetate (TPA). UA was also evaluated in a combination with resveratrol (Res) for possible combinatorial chemopreventive activity.

All triterpene compounds significantly inhibited skin tumor promotion by TPA. MA and 3-epiCA, were significantly more effective than UA at inhibiting tumor development. Topical pretreatment with all of these compounds significantly inhibited epidermal proliferation induced by TPA, however, CA, 3-epiCA and MA were more effective than UA. All compounds reduced skin inflammation and inflammatory gene expression induced by TPA, however, 3-epiCA and MA were identified to be more effective than UA. Finally, the ability of these compounds to alter epidermal signaling pathways associated with skin tumor promotion by TPA was also evaluated. The greater ability of 3-epiCA

and MA to inhibit skin tumor promotion was associated with greater reduction of Cox-2 and Twist1 proteins and inhibition of activation of IGF-1R, Stat3 and Src.

The effect of combining UA + Res for combinatorial inhibitory effects on skin tumor promotion were also examined. The combination of UA + Res produced a greater inhibition of TPA-induced epidermal hyperproliferation, epidermal inflammatory signaling, and inflammatory gene expression when compared to UA or Res alone. Furthermore, NF-κB, Egr-1, and AP-1 DNA binding activities following TPA treatment were dramatically decreased by the combination of UA + Res. Treatment with UA + Res during skin tumor promotion by TPA produced greater inhibition of tumor multiplicity and tumor size than with either agent alone.

Collectively, the current data demonstrate that UA and related triterpenes as well as the combination of UA + Res inhibited skin tumor promotion by TPA via effects on multiple cellular and biochemical/molecular mechanisms associated with this process similar to calorie restriction. Of the tritperpenes tested, 3-epiCA and MA were the most active. Furthermore, the favorable anti-tumor promoting effects of combining UA + Res suggest that phytochemical combination therapy may be a more efficacious strategy for cancer chemoprevention.

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List of abbreviations

°C	Celsius
μg	microgram
μl	microliter
ADP	Adenosine diphosphate
Akt	Protein kinase B
AMPK	5'AMP-activated protein kinase
APC	Adenomatous polyposis coli
ARE	Antioxidant response element
ATG5	Autophagy protein 5
ATP	Adenosine triphosphate
BK5	Basal keratinocyte 5
BrdU	Bromodeoxyuridine
CD3	Cluster of differentiation 3
CD45	Cluster of differentiation 45
CDK2	Cyclin-dependent kinase 2
dNTP	Deoxyuridine nucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinases
HCl	Hydrogen chloride
IKK	IκB kinase
IL	Interleukin

JNK1/2	c-Jun N-terminal kinase 1/2
LC3B	Microtubule-associated protein 1 light chain 3B
LKB1	Liver kinase B1
MAPK	Mitogen-activated protein kinase
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NaCl	Sodium chloride
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
PARP	Poly (ADP-ribose) polymerase
PDCD4	Programmed cell death 4
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC	Protein Kinase C
RTK	Receptor tyrosine kinase
RNA	Ribonucleic acid
S	Serine
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SP1	Specificity protein 1
SP3	Specificity protein 3
SriT1	NAD-dependent deacetylase 1
STAT3	Signal transducer and activator of transcription 3
SV-40	Simian vaculoating virus 40
T	Threonine

TNF-α	Tumor necrosis factor α
TRAF3	TNF receptor-associated factor 3
Y	Tyrosine

Chapter 1: Introduction and background

1.1 Cancer chemoprevention

According to the American Cancer Society, there will be an estimated 1,665,540 new cancer cases diagnosed and 585,720 cancer deaths in the US in 2014. In addition, cancer remains the second most common cause of death in the US, accounting for nearly 1 of every 4 deaths [1]. Even though there is no magic cure to conquer cancer, many types of cancers can be mitigated. Cancer risks can be reduced by eliminating or minimizing exposure to carcinogens. In addition, it is estimated that more than one third of cancer deaths in the United States can be avoided by controlling nutritional factors and diet [2, 3]. Evidence suggests that dietary components can reduce cancer risk. For example, epidemiological studies have shown that fruit and vegetable intake significantly reduced many types of cancers including, lung, colon, breast, cervix, esophagus, stomach, oral cavity, pancreas, and bladder, and ovary [4]. Thus, preventing cancer by controlling the diet may be an effective strategy for controlling cancer.

Given a constant increase in cancer incidence and limitations in cancer treatment, there has been increased interest in studying cancer prevention. In the mid-1970s, Michael Sporn created the term ‘chemoprevention’, which is defined as the use of natural or synthetic agents to reverse, inhibit or slow the beginning or later stages of carcinogenesis. For the last several decades, a number of cancer chemopreventive agents have been identified and shown to inhibit either tumor initiation and/or promotion [5, 6]. Compounds such as ellagic acid and indole-3-carbinol inhibit cancer initiation by pro-carcinogens which are converted to ultimate carcinogens via metabolism and are termed “blocking agents” [6]. The mechanisms of blocking

agents in cancer prevention may involve inhibition of direct interaction of pro-carcinogen with DNA, enhancement of detoxification of pro-carcinogen, and decrease in metabolism of pro-carcinogen to be activated [6-8]. Also, blocking agents may scavenge free radicals, alter DNA repair, and modulate DNA methyl transferases [9]. Cancer suppressing agents inhibit later stages of carcinogenesis including tumor promotion and progression. They suppress signal transduction pathways (e.g., EGFR, NF-κB, AP-1, β-catenin, and STAT3) which are often dysregulated by tumor promoters [6, 9]. In addition, mechanisms of the tumor suppressing agents include decreasing inflammatory gene expression (e.g., chemokines, COX-2, IL-12, TNF-α, and IL-6), inhibition of cell proliferation, and induction of apoptosis in pre-neoplastic lesions [6, 9]. Recently, emerging evidence has suggested that activating AMPK and decreasing mTOR signaling pathways may be a good strategy for cancer chemoprevention by suppressing agents [10-12].

1.2 Two-stage skin carcinogenesis model

The two-stage skin carcinogenesis model is a chemically-induced model of epithelial carcinogenesis that enables evaluation of all stages of tumor development, including tumor initiation, promotion, and progression [13, 14]. This well-established model for studying sequential development of tumors can be used to evaluate cancer chemopreventive agents for their effects on the carcinogenesis process.

In the two-stage skin carcinogenesis protocol, a single topical application of a carcinogen induces irreversible DNA mutation in H-ras and K-ras genes in keratinocyte stem cells [14-17]. DMBA (7,12-dimethylbenz[a]anthracene) is the most common initiator used in the two-stage skin carcinogenesis protocol. It is believed

that keratinocyte stem cells in the bulge region of the hair follicles are the primary cellular target for the initiation stage [18].

After initiation, repeated topical application of a tumor promoting agent (i.e., TPA) leads to skin tumor development. During this promotion stage, the number of nucleated cell layers and epidermal thickness are increased [14, 19, 20] referred to as hyperplasia. In addition, keratinocyte proliferation is significantly increased. Initiated cells progress to clonal outgrowths of skin and eventually become papillomas [14, 19, 20]. In the progression stage, papillomas convert to squamous cell carcinomas as early as 20 weeks of tumor promotion with additional stochastic genetic change, loss of heterozygosity, aneuploidy, and dysplasia. The malignant carcinoma have the capacity to invade the basement membrane and to metastasize to lymph nodes and lungs [14, 21] (Figure 1-1).

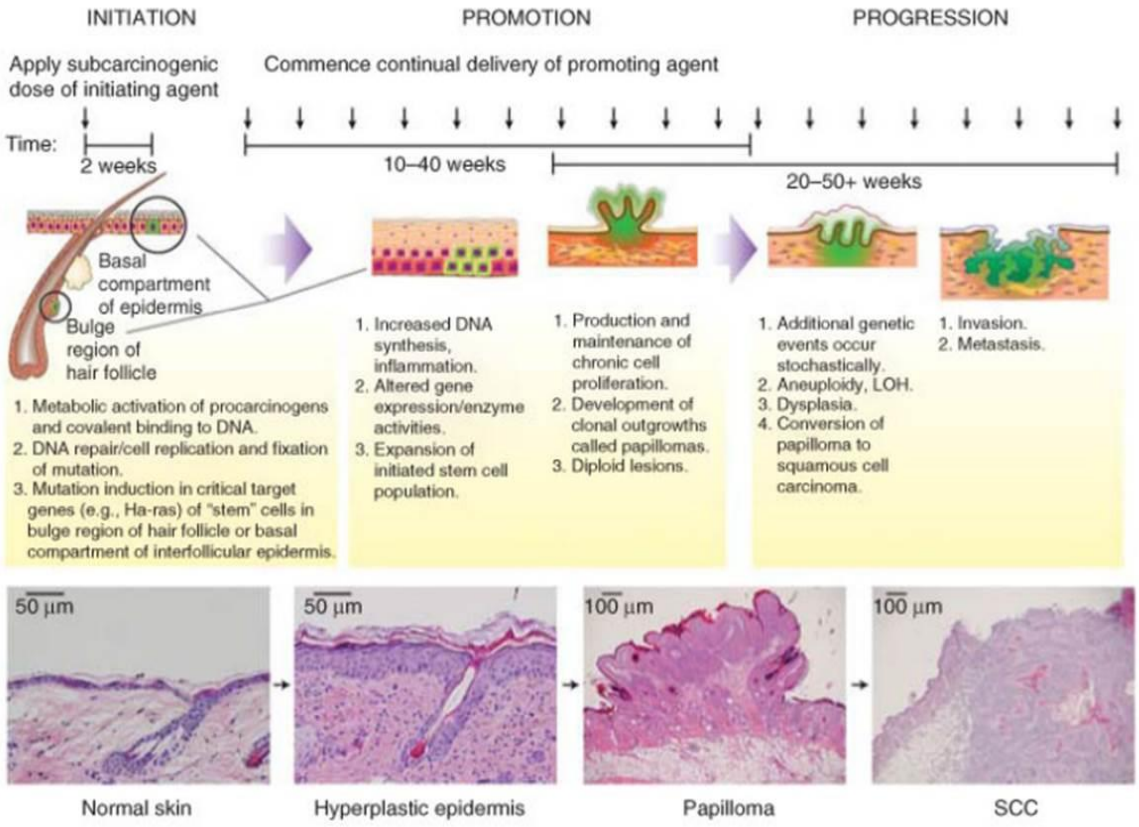


Figure 1-1. **Two-stage model of skin carcinogenesis in mice.** During initiation, topical application of a sub-carcinogenic dose of a mutagenic agent induces mutations in target genes in keratinocyte stem cells. Repeated topical application of a promoting agent begins 2 weeks after initiation and continues for the duration of the study. Papillomas begin to arise after ~6-12 weeks of promotion and a fraction begin to convert to squamaous cell carcinoma (SCC) after ~ 20 weeks. Representative H&E stained sections of normal skin, hyperplastic skin, a papilloma and SCC are presented. All mice were handled in accordance with institutional and national regulations. Adapted from *Nature Protocol* [14] : Abel, E.L., *et al*., Multi-stage chemical carcinogenesis in mouse skin: fundamentals and applications. *Nat Protoc*, 2009. 4(9): p. 1350-62.

1.2.1 Cellular and biochemical mechanisms of skin tumor promotion

During skin tumor promotion, activation of cellular mechanisms involving induction of a sustained proliferation and skin inflammation (e.g., dermal infiltration of inflammatory cells) are critical for skin carcinogenesis. Maintenance of sustained proliferation and inflammation as well as activation of oncogenic signaling pathways is required. Signaling pathways known to be upregulated by tumor promoters include growth factor receptor signaling pathways, cell cycle signaling, and inflammatory signaling pathways. [13, 14, 21].

Hyperproliferation and infiltration of inflammatory cells

During the tumor promotion stage, repeated topical treatments of TPA produce and maintain chronic epidermal cell proliferation [13]. Initiated cells show a differential response to the mitogenic stimulant, TPA, and clonally expand to form pre-malignant papillomas [13, 14, 21]. Another important aspect of tumor promotion is chronic inflammation. Secretion of pro-inflammatory molecules resulting from TPA treatment recruits inflammatory cells such as mast cells, monocytes, leukocytes, T-lymphocytes, B-lymphocytes, and macrophages into the dermis [22, 23]. The number of inflammatory cells increase in the dermis and also promote tumor growth by producing growth factors, cytokines, and chemokines [22, 23].

Receptor tyrosine kinases – EGFR and IGF-1R

i) Epidermal growth factor receptor (EGFR)

Epidermal growth factor (EGF) is a mitogen that binds to EGFR and activates growth signaling pathways involved in proliferation of epidermal keratinocytes [21]. Activated EGFR signaling pathways due to upregulation of its ligands and dysregulated EGFR

expression are observed in skin cancer as well as other cancers including lung, breast and ovarian cancers [24-26] .

The ErbB family is a transmembrane receptor tryrosine kinase family that includes EGFR (or ErbB1), ErbB2, ErbB3, and ErbB4. This receptor family can form homo- or heteromiders upon ligand binding to the extracellular domain and activate the kinase catalytic domain resulting in activation of downstream signaling through phosphorylation of other kinases, adaptor proteins or transcriptional factor [26]. Multiple ligands can activate the EGFR pathway including EGF, transforming growth factor-a (TGF-α), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, and betacelullin [27, 28]. The ligand binding to receptor allows EGFR form a homo- and/or heterodimers with ErbB2 and induces autophosphorylation of tyrosine kinases in the cytoplasmic tails [27, 28].

EGFR is constitutively upregulated in skin papillomas and carcinomas induced by the two-stage protocol [29]. Multiple applications of tumor promoters (e.g., TPA or chrysarobin) increases the expression of multiple ligands such as TGF-α, EGF, and amphiregulin which activates the EGFR signaling pathway during tumor promotion (Figure 1-2) [29]. Transgenic mice overexpressing TGF-α are more susceptible to skin tumor development while mice with a spontaneous inactivating mutation in TGF-α gene were resistant to tumor development using the two stage skin carcinogenesis protocol [30-32]. Similarly, ErbB2 transgenic mice are highly sensitive to TPA-induced epidermal hyperproliferation and two-stage skin carcinogenesis [33]. Also, the EGFR/erbB2 dual inhibitor, GW 2974, inhibited TPA-induced skin tumor

promotion in both BK5-erbB2 mice and wild-type mice [33].

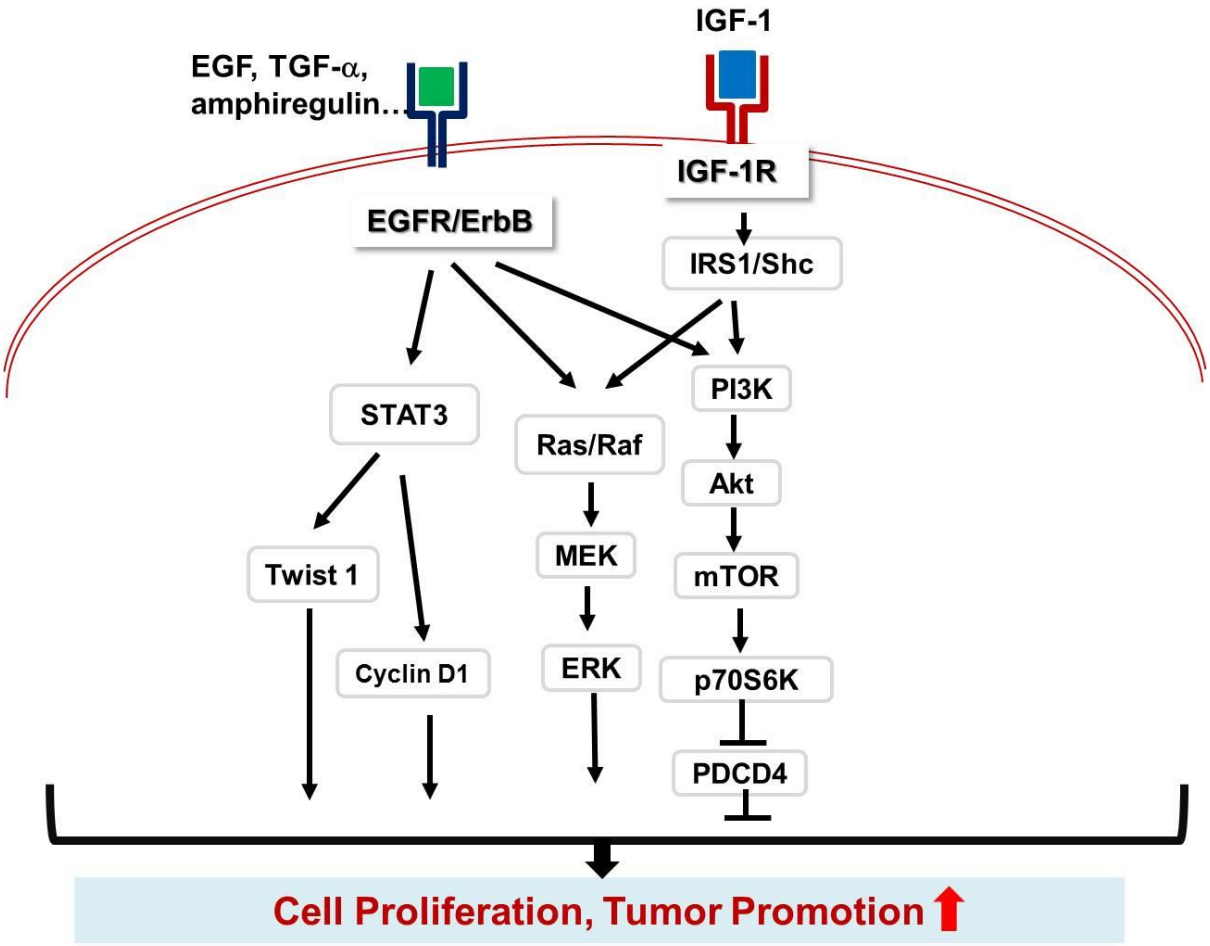


Figure 1-2. Growth factor signaling pathways critical in skin tumor promotion.

ii) Insulin-like growth factor-1 receptor (IGF-1R)

IGF-1 is a polypeptide with structure similar to proinsulin. IGF-1 binds to its receptor (IGF-1R) which is a receptor tyrosine kinase [34-36]. IGF-1 binding to the extracellular α subunit of IGF-1R results in autophosphorylation of the cytoplasmic tyrosine kinase domain in the transmembrane β subunit which leads to phosphorylation of a number of target proteins that bind to specific phosphorylated tyrosine residues, including insulin receptor substrate-1 (IRS-1), IRS-2, Shc, and Crk (see again Figure 1-2) [34-36]. Activated IRS-1 and /or Shc propagate signaling through PI3K/Akt/mTOR or Ras/Raf/MAPK pathways for anti-apoptosis, protein synthesis, cell proliferation, and cell survival [34-36].

The importance of IGF-1R signaling has been emphasized in various cancer models including, lung, breast, and colon. In the mouse skin model, the mRNA level of IGF-1 transcripts was found to be upregulated in both skin papillomas and carcinomas while very low levels of the mRNA were detectable in normal skin [37]. Accumulated data have shown that IGF-1 is important for both initiation and promotion in skin tumorigenesis [38-41]. For example, BK5.IGF-1 transgenic mice overexpressing IGF-1 in the epidermis showed epidermal hyperplasia and hypersensitivity to two-stage carcinogenesis [39]. HK1.IGF-1 mice produced rapid tumor development in response to diverse classes of tumor promoters including chrysarobin, okadaic acid, and benzoyl peroxide when compared to wild-type mice [41]. In addition, spontaneous tumors with or without initiation were observed in BK5.IGF-1 mice [39]. On the other hand, liver-specific IGF-1 deficient (LID) mice, which have reduced circulating IGF-1 levels by ~75%, were less susceptible to two-stage carcinogenesis [40].

Overall, these data suggest that activation of receptor tyrosine kinsases such as EGFR and IGF-1R is important for tumor promoter-induced epidermal hyperproliferation and skin tumor promotion. Also, intervention of these signaling pathways might be a good strategy for cancer prevention.

Proinflammatory cytokines

Cytokines are small proteins that mediate immune response and control various cellular functions including proliferation, differentiation and cell survival/apoptosis [42]. Cytokines are produced in response to inflammatory stimuli by a variety of cells of both the innate (e.g., monocytes and macrophages) and adaptive (e.g., T- and B-cells) immune systems. In addition, epithelial cells can produce cytokines in response to such stimuli. Depending on the tumor microenvironment, some cytokines can induce anti-tumorigenic responses. However, under chronic inflammation conditions, they can cause cell transformation, malignancy, and an imbalance between anti-inflammatory cytokines (e.g., IL-10, IL-4, IL-13 and IL-5) and pro-inflammatory cytokines (IFNγ, IL-1, IL-6 and TNF-α) which can lead to tumor development [43, 44].

Cytokines including TNF-α, IL-1, IL-6, IL-17, IL-22, and IL-23 can contribute to skin tumor development [21]. Their expression level is increased in response to tumor promoters such as TPA and okadaic acid, and binding to their receptors leads to propagation of signaling pathways involved in cell apoptosis, survival, inflammation, proliferation, and differentiation for tumor development [21]. For example, tumor necrosis factor-α (TNF-α) binds to homotrimeric transmembrance receptors, TNFR1

and TNFR2 [45, 46]. Ligand-bound TNFRs recruit various adaptor proteins such as TNFR1-associated death domain protein (TRADD), receptor interacting protein kinase (RIP), and TNFR-associated factor-2 (TRAF2) [46]. The recruited proteins then activate the following signaling pathways; 1) FADD-Caspase-8/10 for apoptosis, 2) IKK/IκB/NF-κB pathway for inflammation and cell survival, and 3) GCK/MKK7/4/JNK-AP-1 or ROS/ASK1/MKK7/4/JNK/AP-1 for gene expression, cell proliferation, and differentiation (see Figure 1-3) [46].

Interleukins are also important for tumor development. They are produced by many cell types including leukocytes [47]. Several interleukins have been shown to have a clear role in skin tumor promotion. The expression level of IL-1α and IL-β are increased in response to TPA treatment *in vitro* and *in vivo* [48-50]. IL-1 binds to IL-1R which recruits a transmembrane receptor accessory protein (IL-1RAcP) to propagate signals through IL-1R-associated kinase (IRAK)-NF-κB signaling for inflammation (see Figure 1-3) [51, 52]. In the skin carcinogenesis model, mice overexpressing IL-1α in basal keratinocytes develop spontaneous skin inflammation and dermal neutrophil infiltration [53]. Also DMBA treatment increased mRNA levels of IL-1α and it is also upregulated in both skin papillomas and carcinomas when compared to normal skin [54]. IL-1Ra is an endogenous receptor antagonist and is increased in TPA-induced mouse keratinocytes *in vitro* as well as both in papillomas and carcinomas [54]. In addition, La *et al.* reported that even though overexpression of IL-1Ra in a skin carcinoma cell line (icIL-1Ra-JWF2 cells) did not change IL-1α mRNA levels, these transfected cells had a decreased growth rate compared with parental JWF2 cells [55]. Conversely, IL-1Ra null mice are more susceptible to skin tumorigenesis by DMBA/TPA when compared to wild-type mice,

suggesting that IL-1 signaling pathway plays a critical role in skin tumor promotion [56].

In addition to IL-1, cytokines including IL-17, IL-22 and IL-23 are also regarded as important inflammatory cytokines for skin tumor promotion [21]. IL-22 and IL-23 bind to their receptors IL-10R/IL-22R and IL-12Rβ1/IL-23R, respectively, and subsequently activate JAK/STAT3 pathways [57, 58]. Binding of IL-17 to IL-17RC/IL-17RA leads to activation of TRAF3/6-IKK-IκB-NF-κB pathway which is often dysregulated in skin tumor promotion [21]. Nardinocchi *et al*. reported that IL-22 and IL-17 promotes tumor progression in human melanoma skin cancer and increased expression of IL-22 and IL-17 accelerated tumor growth in nude mice injected with squamous cell carcinoma cells [59]. IL-23p19 null mice have been shown to be resistant to two-stage skin carcinogenesis and IL-17 is highly expressed in the hyperplastic skin after TPA treatment in mice [60]. In addition, Wang *et al.* have reported that IL-17 enhanced skin tumor development and disruption of IL-17 significantly inhibited skin carcinogenesis in mice using skin carcinogenesis protocol [61].

Collectively, these data demonstrate that expression of inflammatory cytokines such as TNF-α, IL-1α, IL-1β, IL-6, IL-17, IL-22, and IL-23 are elevated during tumor promotion and upon binding to their receptors activate oncogenic signaling pathways involved in skin tumor development.

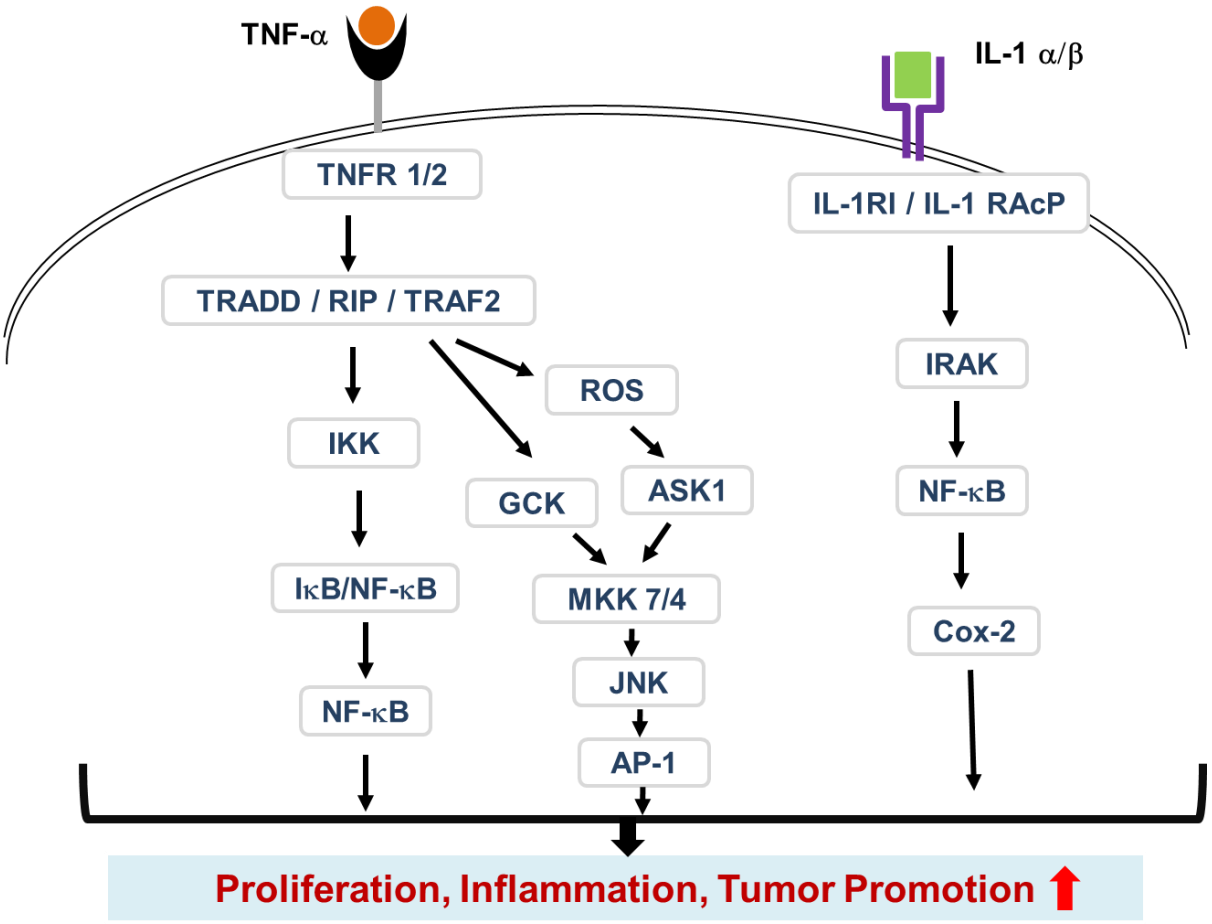


Figure 1-3. Signaling pathways of TNF-α and IL-1 α/β important in skin tumor promotion

Cyclooxygenase-2 (Cox-2)

Cyclooxygenases are enzymes that catalyze the synthesis of prostaglandins from arachidonic acid [62]. There are two isoforms of cyclooxygenase, Cox-1 and Cox-2. While the expression of Cox-2 is inducible by tumor promoters, Cox-1 is a house keeping enzyme that is constitutively expressed in many tissues [62]. Cox-2 has been shown to be overexpressed in cancer tissues such as colon, liver, breast, lung, pancreas, bladder and stomach [63]. In addition, a number of studies have shown that level of Cox-2 is elevated during skin tumor development [64]. Multiple pathways are suggested to increase Cox-2 levels by regulating upstream transcription factors. Since release of acrachidonic acid from the membrane is induced by PKC activation, it is not surprising that TPA-induced PKC activation mediates Cox-2 expression [65-67]. Also Sp1/Sp3-p38 MAPK activation mediates EGF/EGFR-induced Cox-2 induction [68]. Several transcription factors including NF-κB, CCAAT/enhancer-binding protein (C/EBP), and upstream stimulatory factors (USFs) directly bind to the Cox-2 promoter region [69-71]. Also TPA treatment results in upregulation of Cox-2 expression in mouse keratinocytes *in vitro* [64]. Several *in vivo* studies have shown that Cox-2 knock-out mice were less sensitive to two-stage skin carcinogenesis when compared to wild-type mice and, topical treatment with selective pharmacological Cox-2 inhibitors (e.g., Celecoxib) inhibited skin tumor promotion [72-74]. On the other hand, transgenic mice overexpressing Cox-2 in the basal layer of the epidermis were more susceptible to two-stage skin carcinogenesis [75, 76]. Overall, these data demonstrate that Cox-2 has a clear role as an oncogenic protein in skin tumorigenesis and identification and development of safe and effective Cox-2 inhibitors is an ongoing goal in many laboratories.

NF-κB and AP-1

Nuclear factor κB (NF-κB) and activator protein 1 (AP-1) are important transcription factors that are involved the expression of genes related to inflammation, embryonic development, proliferation, oncogenesis, and apoptosis [77-79]. Dysregulation of NF-κB is related to anti-apoptosis and proliferation in malignant cells [78]. Stimuli such as reactive oxygen species (ROS), lipopolysaccharide (LPS) and cytokines (e.g., TNF-α, IL-1, and IL-6) have shown to activate NF-κB, and many cancer chemopreventive agents have inhibitory effects on NF-κB activation [79]. NF-κB consist of p50 and p65 in its resting stage and is inhibited by inhibitory factor IκB in the cytosol of cells [78]. However, with stimuli such as free radicals, UV and cytokines, IκB undergoes phosphorylation and ubiquitination for degradation and NF-κB can be activated by phosphorylation. NF-κB then translocates into the nucleus and binds to κB-binding site for activation of numerous genes. AP-1 is a homo- or heterodimeric protein consisting of proteins belonging to jun (c-jun, junB, and junD) and fos (c-fos, fosB, fra-1, and fra-2) family [80]. Transcription of jun and fos is regulated by external stimuli such as UV, growth factors and cytokines, and jun-jun or jun-fos dimers bind to AP-1 consensus DNA binding elements in target genes to regulate expression [77, 80, 81].

A considerable number of studies have shown that NF-κB and AP-1 play an important role in the development of skin tumors in the two-stage model. For example, AP-1 potentially promotes skin cancer development via its control of keratinocyte survival and differentiation [82]. Young *et al*. reported that transgenic mice having a transactivation mutant *c-jun* under the control of the human K14

promoter exhibited dramatic inhibition of papilloma induction in the two-stage skin carcinogenesis protocol [83]. Expression of NF-κB p50 and DNA binding activity of NF-κB p65/p50 are increased in mouse skin papillomas and squamous cell carcinomas [84]. In addition, Kim *et al*. have shown that mice with keratinocyte-restricted p65/RelA deficiency have reduced sensitivity to two-stage skin carcinogenesis by protecting keratinocytes from DNA damage-induced death and facilitating the establishment of a tumor-nurturing proinflammatory microenvironment [85].

STAT3

Signal transducer and activator of transcription 3 (STAT3) is one of six mammalian STAT family members that have been identified [86, 87]. STAT3 can be activated through cytokine receptors (e.g., IL-6, IFNγ), G-protein coupled receptors, growth factor receptors (e.g., EGFR), or non-receptor tyrosine kinase (e.g., c-src) [29, 86, 87]. Once activated, STAT3 will homodimerize and then translocate to the nucleus where it will bind to DNA and activate transcription of genes involved in regulation of anti-apoptosis, cell proliferation, survival, and angiogenesis [29].

The role of STAT3 in epithelial carcinogenesis has been well studied [29, 87]. Activated STAT3 in epidermis by chemical tumor promoters or UVB regulates the expression of target genes such as c-myc, Bcl-xL, survivin, Bcl-2, cyclin D1, c-Myc, c-fos, VEGF, and twist that are involved in stem cell maintenance, survival, proliferation and EMT [29, 86, 87]. Several studies have shown that STAT3 is important for skin tumor promotion, and ablation of STAT3 leads to reduction in epidermal hyperproliferation, inhibition of tumor development, and decreased

expression of genes involved in regulation of cell cycle (e.g., cyclin D1 and c-myc) in chemically-induced skin carcinogenesis [86, 88-93]. Conversely, transgenic mice expressing a constitutively active form of STAT3 in the epidermis displayed increased malignant progression during two-stage carcinogenesis [92]. In this study, the transgenic mice developed skin tumors more rapidly and with higher number of tumors when compared to WT mice. Furthermore these tumors became squamous cell carcinomas bypassing the premalignant stage of skin carcinogenesis. Overall, these data suggest that STAT3 plays an important role in epithelial carcinogenesis and given the strong evidence for STAT3 involvement in all stage of tumorigeneis, it represents an attractive target protein for cancer chemoprevention.

AMPK

The 5′-AMP-activated protein kinase (AMPK) is an energy sensing enzyme that contains α-, β-, and γ-subunits and once activated, AMPK phosphorylates and regulates key enzymes to regulate cellular lipid and protein metabolism in response to stimuli such as exercise and changes in cellular ADP/ATP ratio [94-96]. It is activated primarily by two molecules, tumor suppressor LKB1 and Ca^{+2}/calmodulin-dependent protein kinase β (CaMKKβ) [97-99]. The reduced ratio of ADP/ATP leads to LKB1 activation, which results in phosphorylation of AMPK-α on threonine 172 [94, 98, 99]. Also increases in intracellular Ca^{+2} level can activate CaMKKβ kinase activity which leads to activate phosphorylation and activation of AMPK-α [99]. Emerging evidence indicates that AMPK is a promising metabolic tumor suppressor and a target for cancer prevention and therapy. The AMPK pathway is negatively correlated with the mTOR and NF-κB pathways and increased activation of AMPK mediates an

autophagic signaling pathway through Ulk1. In addition, AMPK signaling also interacts with two critical tumor suppressors, p53 and ATM to coordinate metabolic checkpoints and DNA repair response [100, 101]. Canto *et al*. showed that AMPK activation increases intracellular NAD^+ and subsequently stimulates SirT1 activity [102, 103]. Interestingly, Lan *et al*. have reported that SirT1 can act as an upstream activator of LKB1 by showing that SirT1 deacetylates LKB1 kinase which subsequently increased its activity [104]. Since LKB1 is an upstream kinase of AMPK, the SirT1-LKB1 pathway increased AMPK activation [104]. These data suggest that there might be a positive feedback loop between AMPK and SirT1 for potentiation of their activation to control energy balance effectively. Even though limited information is available for the role of AMPK as a tumor suppressor, *Wu et al.* reported that AMPK activation is decreased in human and UVB-induced mouse squamous cell carcinoma when compared to normal skin [105]. Also, in this study, topical treatment with AMPK-α activator, AICAR and metformin, reduced UVB-induced skin tumorigenesis. More recently, our group reported that metformin, which is known to activate AMPK-α, significantly inhibited skin tumor promotion by TPA when given in drinking water [11]. Furthermore, topical treatment of resveratrol, a calorie restriction mimetic and SirT1 activator, suppressed skin tumorigenesis [106-108]. Overall, these data suggest that AMPK might act as a tumor suppressor and identification and development of safe AMPK activators might be a good novel strategy for cancer prevention.

1.3 Calorie restriction and skin tumorigenesis

Calorie restriction (CR) is a reduction in calorie intake (usually in the range of 20~40 %) without malnutrition or lack of mineral or vitamin needs. CR is considered

the most consistent non-pharmacological factor that increases lifespan and decreases detrimental biological functions in model organisms [109]. Recently, numerous studies have shown that CR inhibits tumorigenesis in various experimental models [110-119]. A number of studies reported that CR suppresses tumor promotion, however, no effects were observed on either initiation or progression using the well-established two-stage skin carcinogenesis model [113, 120, 121]. Proposed mechanisms for the inhibitory effect of CR on skin tumor promotion are as follows: i) 10~40% CR (low fat or low carbohydrates) inhibits TPA-induced tumor promotion due to reduced PKC activity in epidermal cells [112, 113]; ii) 40 % CR blocks TPA-induced AP-1 and ERK1/2 activation in skin tumor promotion [122]; iii) 30 % CR decreases TPA-activated epidermal signaling of EGFR & IGF-1R and their crosstalk as well as PI3K/Akt/mTOR signaling in mouse epidermis [40, 121, 123]; iv) CR has anti-tumor promoting effect and the effect, at least in part, is mediated through an Nrf2- mechanism [124, 125].

CR delays the onset of aging-related chronic disease including tumorigenesis as mentioned above. Even though CR has been shown to benefit human health, it is extremely difficult to maintain a life style based on 20~40% CR for a large portion of the population. Therefore, it is not surprising that considerable effort has been placed on developing CR mimetics by focusing on identification of pathways that regulate the beneficial effects of CR without commitment of major lifestyle changes.

1.4 *Perilla (P.) frutescens*

P. frutescent belongs to the annual mint family and is an edible plant (Figure 1-4) frequently used in Asian countries including Korea, Japan and China. Since it has a

pleasant flavor and taste, it is widely used in Korean food ingredients. For example, perilla leaves can be added to fish, rice, soup, and vegetables, and it can be pickled for many dishes. *P. frutescens* is not only used as a food ingredient but also for medicinal preparations due to its anti-allergic and anti-inflammatory effect. Banno *et al.* have reported that a number of pentacylic triterepenes are present in *P. frutescens* including ursolic acid (UA), oleanolic acid (OA), corosolic acid (CA), 3-epi-corosolic acid (3-epiCA), maslinic acid (MA), 3-epimaslinic acid (3-epiMA), tormentic acid (TA), pomolic aicd (PA), hyptadienic acid (HA), and augustic acid (AA) (Figure 1-5) [126]. These compounds are triterpenoid carboxylic acids with the molecular formula $C_{30}H_{48}$ having six isoprene units and are synthesized in *P. frutescent* by cyclization of squalene. Banno *et al. [126]* reported that all of these compounds exhibited some anti-inflammatory activity against TPA-induced ear edema. Also TA was shown to inhibit skin tumor promotion by 12-O-tetra-deacnoylphorbol-13-acetate (TPA) in this study [126]. In other studies both UA and OA have been shown to inhibit skin tumor promotion by TPA [107, 127, 128]. Even though several of the compounds found in *P. frutescens* have been shown to possess anti-tumor promoting and cancer chemopreventive activity, only limited information is available on the other triterpenes and on the mechanisms of action of these compounds.

Figure 1-4. *Perilla (P.) frutescens*

P. frutescent var. crispa (Labiatae) belongs to the annual mint family. It is edible and is used as a medicinal plant in many Asian countries. There are two chemo-types: red perilla and green perilla. The photo is of green perilla.

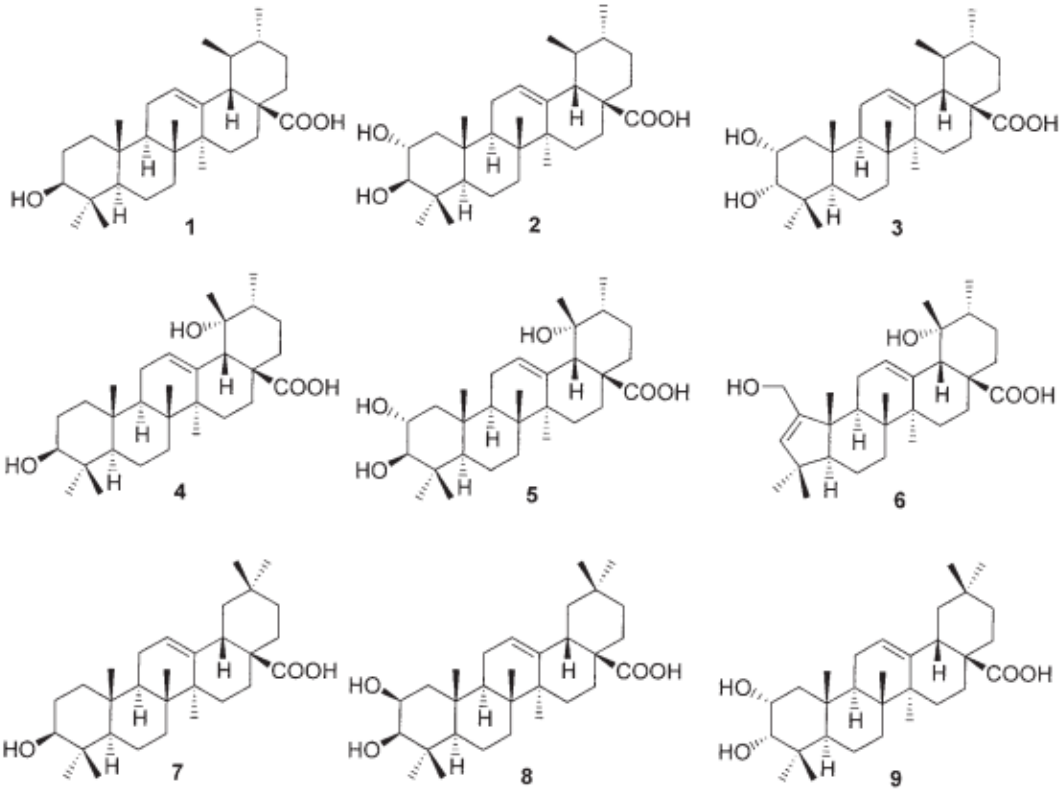


Figure 1-5. Pentacyclic triterpenes found in *P. frutescens*

1, Ursolic acid; **2**, Corosolic acid; **3**,3-epi-corosolic acid;**4**, Pomolic acid; **5**, Tormentic acid; **6**, Hyptadienic acid; **7**, Oleanolic acid; **8**, Augustic acid; and **9**, 3-epi-maslinic acid

Adapted from *Bioscience, Biotechnology, and Biochemistry [126]:* Banno, N., *et al*., *Triterpene acids from the leaves of Perilla frutescens and their anti-inflammatory and antitumor-promoting effects. Biosci Biotechnol Biochem*, 2004. **68**(1): p. 85-90.

1.4.1 UA and related triterpenes found in *P. frutescens*

UA is a natural pentacyclic triterpenoid carboxylic acid found in *P. frutescen* (Japanese basil) as well as rosemary, apples, elder flowers, and many other plants. It has been reported to have a broad range of pharmacological properties and is regarded as one of most potent chemopreventive agents for cancer. UA has been shown to have apoptotic, anti-inflammatory and anti-tumorigenic effects in various cancer models including prostate, ovary, small intestine, stomach, and skin [126, 127, 129]. Further studies have revealed that UA has a broad-spectrum of anti-carcinogenic effects including prevention of DNA damage, inhibition of EGFR/MAPK signaling, inhibition of angiogenesis, activation of apoptotic signaling pathways, and inhibition of Akt-mTOR, NF-κB, Cox-2, and STAT3 signaling pathways [129, 130]. Although several studies have reported that UA inhibited TPA-induced inflammation, hyperplasia and tumor promotion in mouse skin [107, 126, 127], its inhibitory mechanism is not fully understood. Recently, several studies reported that UA also has an anti-obesity effect and mimics some of the effects of CR by modulating Akt/mTOR signaling pathways [131-133]. UA has also been shown to activate the LKB1/AMPK pathway for inhibition of adipogenesis [134].

Together with UA, OA is also found in *P. frutescens*. It is also abundant in ginseng root and olive oil. UA and OA are often found together in plants and possess similar pharmacological properties [129, 135-137]. OA exhibits unique properties including anti-inflammatory, gastro-protective, wound-healing, and anti-microbial effects [138, 139]. In addition, several studies have shown anti-cancer effects of OA in both *in vitro* and *in vivo* studies [127, 140]. For example, OA inhibits proliferation and induces apoptosis in many cancer cell lines including breast, lung, and skin [141-

144]. It has been shown to have antioxidant capacity by activating Keap1-Nrf2-ARE pathways in hepatocellular cancer cells [145]. Two, 5, and 10 μmol of OA pretreatment inhibited TPA-induced expression of ornithine decarboxylase, c-fos, and c-Jun [140] and 41 nmol of OA given as a pretreatment inhibited TPA promotion in the two stage skin carcinogenesis assay [127]. In another study, OA inhibited growth of hepatocellular carcinoma cells by inducing p53-ERK-mediated cell cycle arrest and mitochontrial-dependent apoptosis in BALB/C mice [146]. Furtado *et al*. reported OA inhibited 1,2-dimethyhydrazine induced colon carcinoma in rats [147].

Maslinic acid (MA), also known as crategolic acid, has been isolated from *P. frutescens* as well as other edible plants such as olive fruit, spinach, eggplant, mustard, basil and legumes [148]. A number of *in vitro* studies have shown MA to possess anti-proliferative, apoptotic, and anti-cancer properties. The anti-proliferative and apoptotic properties of MA have been demonstrated in various cancer cell lines such as colon, liver, bladder, uterus and breast [149-154]. Proposed mechanisms for the anti-cancer effect of MA in *in vitro* experiments include activation of caspase-3, inhibition of Cox-2 expression, suppression of IκB-NF-κB pathway, increased expression of p53, and modulation of JNK1/2 or p38 pathway [148]. Until now, only a few studies have reported the anti-tumor activity of MA using *in vivo* models. Li *et al.* have demonstrated subcutaneous administration of MA (10 and 50 mg/kg) inhibited growth of pancreatic cancer cells in a xenograft mouse model via an increase of apoptosis and reduction of NF-κB-mediated anti-apoptotic genes, including survivin and Bcl-xL [155]. In another study, dietary MA (100 mg/kg of control diet) in APC Min/+ mice reduced colon carcinogenesis by 45 % [156]. In addition, MA inhibited both size and weight of bladder tumors in a xenograft mouse model [152].

CA exists in abundance in the plant kingdom including bananas, loquat, and *P. frutescens*. A number of studies have shown that it has anti-diabetic, anti-oxidant, anti-inflammatory, apoptotic, and anti-cancer activities [157-164]. The anti-cancer effects of CA have not been extensively studied *in vivo*. However, several *in vitro* studies have been reported growth inhibitory effects in many cancer cell lines including gastric, colon, cervix, leukemic and lung [157-163]. Suggested mechanisms for its anti-cancer effects include inhibition of PKC activity [157], induction of cell cycle arrest, activation of apoptotic molecules (e.g., Bax and caspases, 3,8, and 9) [158, 161], regulation of cell cycle proteins (e.g., induction of p27 and inhibition of cyclin D1) [159], deregulation of HER2 downstream pathways (e.g., reduction of activated Erk or Akt) [159], modulation of the AMPK-mTOR pathway [160], inhibition of NF-κB and STAT3 pathway [162], and downregulation of β-catenin [163].

In summary, previous data have demonstrated that UA is an effective chemopreventive agent in animal models. Together with UA, a number of pentacyclic triterpenes found in *P. frutescens*, including, OA, CA, MA appear to also possess anti-carcinogenic properties. However, the mechanisms of their chemopreventive activities are poorly understood and several of the triterpenes found in *P. frutescens* including, AA, 3-epiCA, and 3-epiMA have not been thoroughly studied. In this regard, detailed examination of anti-carcinogenic effect of these compounds is needed. In addition, more information on the exact inhibitory mechanism(s) for this class of compounds is essential for further evaluation of chemopreventive efficacy in humans.

1.5 Resveratrol

Resveratrol (Res) is a phytoalexin which possess antimicrobial and antioxidant properties [165, 166]. Res is synthesized de novo by plants to counteract pathogen infections. It is found in plants such as in grapes, berries, and peanuts. For the past several decades, Res has been extensively studied and shown to have cardiovascular benefit (French paradox), CR-resembling effects, anti-diabetic effects, and anti-cancer effects in mice and humans. In addition, Res is regarded as an effective chemopreventive agent based on numerous preclinical studies [106-108, 167-172]. Res has been shown to inhibit skin tumor promotion and inhibit the growth of many cancer cell lines, including breast, prostate, colon and liver. Several studies have elucidated potential mechanisms for its anti-tumor promoting effects including inhibition of Cox-2, NF-κB, and mTOR signaling pathways in skin [167, 169]. Several reports suggest that Res mimics some of the effects of CR on life span in worms and other model organisms, especially by inhibiting inflammation and mTOR [170, 173]. Res also mimics the effects of CR by increasing SirT1 and AMPK activation [171]. Also Boily *et al*. have suggested that the anti-promoting effect of Res on mouse skin is mediated, at least in part by SirT1 [106]. Although the anti-cancer effects of Res observed both *in vitro* and *in vivo* is widely studied, the exact mechanism of its anti-proliferative effects remains to be elucidated.

1.3 Combination of phytochemicals and chemoprevention

Emerging evidence suggests that combinations of phytochemicals may be a good strategy to achieve greater chemopreventive efficacy [174, 175]. Many phytochemicals appear to have CR mimetic properties (e.g., resveratrol [171]). Because they have relatively low specificity towards single target proteins and low

toxicity when compared with synthetic compounds, combining phytochemicals may be an advantage for cancer prevention. Several studies have shown that combinations of natural compounds can produce potentially synergistic inhibitory effects on tumor formation (e.g., resveratrol + grape seed extract and ellagic acid + grape seed extract) [176-181]. Thus, combining agents may provide the most rational and effective approach to cancer chemoprevention and provide overall effects that more similarly mimic CR.

1.7 Hypothesis and specific aims

The overall goal of this study was to examine the effect of natural compounds that mimic some effects of CR on skin tumor promotion. In particular, we tested the possible CR effects of 7 different triterpenes found in *P. frutescens* on TPA-induced skin tumor promotion. Preliminary data showed that an extract from green perilla leaves inhibited TPA-induced Akt, NF-κB p65, p70S6K, Cox-2, and CDK2. Also the perilla extract partially reversed TPA effect on PDCD4. Based on these preliminary data a series of related pentacyclic triterpenes, including UA from *P. frutescens,* were analyzed. These components were evaluated for their inhibitory effects on TPA-induced epidermal signaling pathways, hyperproliferation, inflammation and skin tumor promotion. Moreover, we also focused on the effects of combined treatment of one of the pentacyclic triterpenes, UA, and Res on TPA-induced skin tumor promotion. Preliminary data had indicated that combining these two agents could produce a greater CR mimetic effect. **The hypothesis to be tested was that triterpenes found in *P. frutescens* possess CR mimetic properties in their ability to inhibit mouse skin tumor promotion. Furthermore, UA in combination with Res will possesses greater CR mimetic activity by modulating a broad**

range of growth factor, inflammatory and possibly other signaling pathways similar to CR to suppress proliferation of initiated keratinocytes and inhibit skin tumor promotion.

The specific aims are as follows:

Specific aim 1: Examine the effect of UA and a series of related pentacyclic triterpenes on epidermal signaling pathways by TPA.

In this aim, a series of pentacyclic triterpenes including UA, OA, AA, CA, 3-epiCA, maslinic acid, and 3-epiMA found in *P. frustescens* were examined for their effects on TPA-induced epidermal signaling pathways important for skin tumor promotion. The pathways analyzed included RTKs (EGFR and IGF1R) Akt/mTORC1, NF-κB, AP-1, p38, JNK1/2, and AMPK.

Specific aim 2: Examine the effect of UA and a series of related triterpenes on keratinocyte proliferation and skin inflammation in relation to their ability to inhibit skin tumor promotion by TPA.

In this aim, we examined the effect of UA and other triterpenes on TPA-induced epidermal hyperplasia, and hyperproliferation. We also examined the effect of these compounds on TPA-induced skin inflammation and inflammatory gene expression. In this Specific Aim, we also evaluated the ability of these triterpenes from *P. frutescens* (except AA) to inhibit skin tumor promotion by TPA during two-stage skin carcinogenesis.

Specific aim 3: Examine the possible combinatorial inhibitory effects of UA

given together with Res on TPA-induced epidermal signaling pathways, epidermal hyperproliferation, inflammation and skin tumor promotion.

In this Specific Aim, we evaluated the effects of combined treatment with UA + Res on epidermal signaling pathways, epidermal proliferation, skin inflammation and inflammatory gene expression induced by TPA compared to either compound given alone. Furthermore, we tested the ability of this combination to produce a combinatorial inhibitory effect on skin tumor promotion by TPA.

Chapter 2. Materials and Methods

Animals and Diets

Female Hsd : ICR (CD-1) and FVB/N mice 6-7 weeks of age were purchased from Harlan Laboratories Inc. (Frederick, MD) and the National Cancer Institute (Frederick, MD), respectively. Mice were group housed for all experiments. For the short-term experiments, mice were fed a regular chow diet. For tumor experiments, mice received either an overweight control diet (D12450B, 10 Kcal% fat; Research Diets Inc.) or an obesity inducing diet (D12492, 60 Kcal % fat; Research Diets Inc) (see diet formulas in Table 2-1).

	10 Kcal% Fat Diet		60 Kcal% Fat Diet	
Product #	D12450B		D12492	
	gm %	Kcal %	gm %	Kcal %
Carbohydrates	67.3	70	26	20
Protein	19.2	20	26	20
Fat	4.3	10	35	60
Total		**100**		**100**
Kcal / gm	**3.85**		**5.24**	
Casein, 30 Mesh	200	800	200	800
L-Cystine	3	12	3	12
Corn Starch	315	1260	0	0
Maltodextrin 10	35	140	125	500
Sucrose	350	1460	68.8	275
Cellulose, BW200	50	0	50	0
Soybean Oil	25	225	25	225
Lard	20	180	245	2205
Mineral Mix S10026	10	0	10	0
DiCalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate, 1 H2O	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
FD&C Yellow Dye #5	0.05	0	0	0
FD&C Blue Dye #1	0	0	0.05	0
Total	**1055.05**	**4057**	**773.85**	**4057**

Table 2-1. Formulas of 10 Kcal% fat and 60 Kcal% fat diets.

Chemicals

UA and OA were purchased from Sabinsa Corporation and Standford Chemicals, respectively. AA, CA, MA, 3-epiCA, and 3-epiMA were prepared as recently described by us [211]. All triterpenes used in the current experiments were > 98% pure. We received Res from Orchid Chemicals & Pharmaceuticals Ltd. DMBA was purchased from Sigma Chemical Co. and TPA was purchased from LC laboratories.

Short-term treatment protocol for the anti-tumor promoting effect of a series of related pentacyclic triterpenes

Mice were treated using a short-term treatment protocol involving 4 applications of TPA. For this protocol, as shown in Figure 2-1, groups of mice (7-8 weeks of age) were shaved on the dorsal skin and then two days later treated twice weekly for two weeks with 0.2 ml acetone vehicle, 2 μmol of UA, OA, AA, CA, 3-epi-CA, MA, and 3-epi-MA 30 min prior to each 6.8 nmol of TPA treatment. Mice were then sacrificed at various times thereafter for collection of epidermal tissue.

Short-term treatment protocol for the anti-tumor promoting effect of the combination of UA + Res

For a number of experiments, mice were treated using a similar short-term treatment protocol involving 4 applications of TPA (Figure 2-2). For these experiments, groups of female ICR mice (7-8 weeks of age) were shaved on the dorsal skin and then two days later treated twice-weekly for two weeks with 0.2 ml acetone vehicle, UA (2 μmol) or Res (2 μmol) 15 min prior to each 6.8 nmol of TPA treatment. For the combination, mice received UA (2 μmol) and Res (2 μmol) 15 min and 30 min prior to TPA treatment, respectively. Mice were then sacrificed at various times thereafter for

collection of epidermal tissue.

A

Group	Pretreatment (-30min)	Treatment
1	Acetone	Acetone
2	Acetone	6.8 mol TPA
3	2 μmol UA	6.8 mol TPA
4	2 μmol OA	6.8 mol TPA
5	2 μmol AA	6.8 mol TPA
6	2 μmol CA	6.8 mol TPA
7	2 μmol 3-epiCA	6.8 mol TPA
8	2 μmol MA	6.8 mol TPA
9	2 μmol 3-epiMA	6.8 mol TPA

B

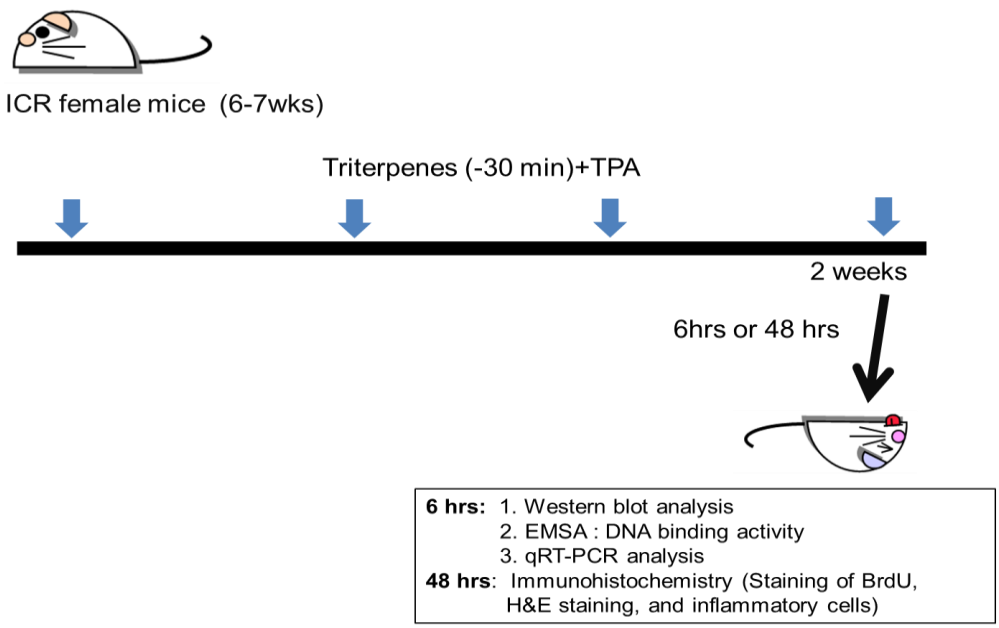


Figure 2-1. Short-term treatment protocol for the anti-tumor promoting effect of a series of related pentacyclic triterpenes

A, treatment groups B, short-term treatment protocol.

A

Group	1st Pretreatment (-30min)	2nd Pretreatment (-15min)	TPA Treatment
1	Acetone	Acetone	Acetone
2	Acetone	Acetone	6.8 mol TPA
3	Acetone	2 μmol UA	6.8 mol TPA
4	Acetone	2 μmol Res	6.8 mol TPA
5	2 μmol Res	2 μmol UA	6.8 mol TPA

B

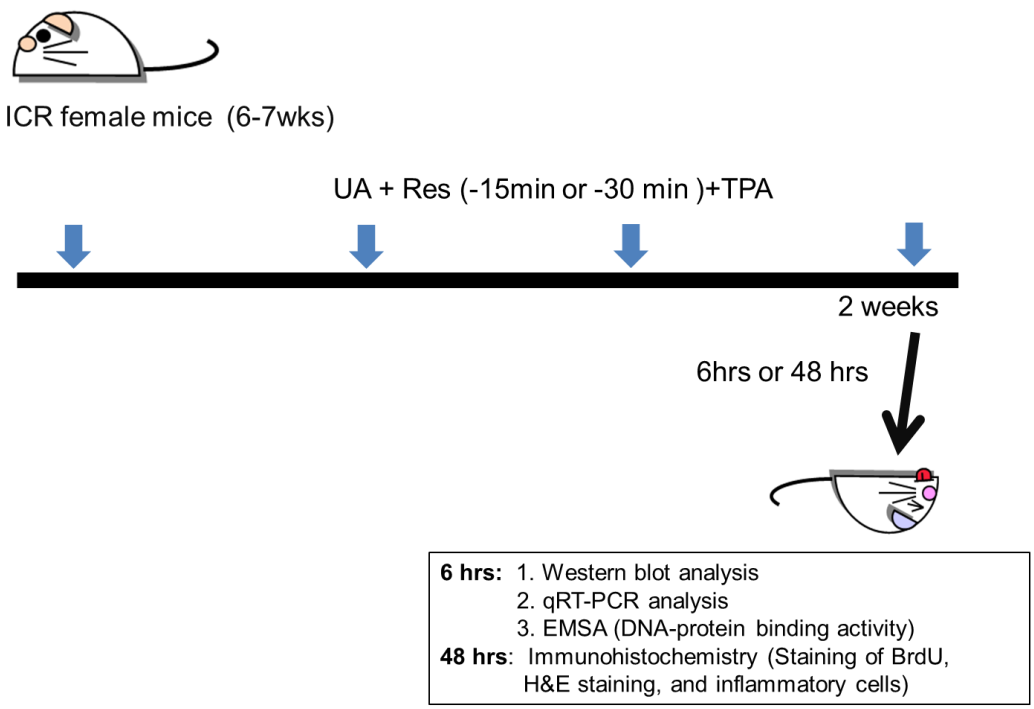


Figure 2-2 Short-term treatment protocol for the anti-tumor promoting effect of combination of a UA + Res

A, Treatment groups B, short-term treatment protocol

Two-stage skin carcinogenesis assays for the anti-tumor promoting effect of pentacyclic triterepens found in *P. frutescens*

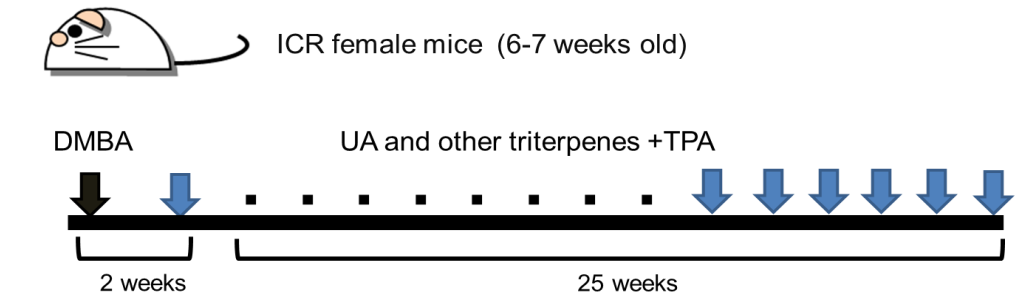
Female ICR mice (n=30/group) 7-8 weeks of age maintained on 10 Kcal % fat diet (overweight diet) were shaved on the dorsal skin and then 48 hrs later initiated with a single topical application of 25 nmol of DMBA in 0.2 ml acetone or acetone vehicle. During tumor promotion, mice received 2 μmol of UA , OA, CA, Epi-CA, MA or Epi-MA 30 min prior to each 6.8 nmol TPA application. TPA was administered twice-weekly for the duration of the experiment (i.e., 23 weeks (Figure 2-3A). Body weight, tumor incidence (percentage of mice with papillomas) and tumor multiplicity (average number of papillomas per mouse) were measured once a week for the duration of the experiment (Figure 2-3A). In addition, the surface area of all detectable papillomas was measured by digital calipers at the termination of the experiment.

Two-stage skin carcinogenesis assays for the anti-tumor promoting effect of a combination of UA + Res

As shown in Figure 2-3B, Female ICR mice (n=30/group) 7-8 weeks of age were shaved on the dorsal skin and then 48 hrs later initiated with a single topical application of 25 nmol of DMBA in 0.2 ml acetone or acetone vehicle . Two weeks after initiation, mice were randomized to receive one of the two experimental diets (overweight control diet and obesity-inducing diet) for 6 weeks before starting treatment with the tumor promoter, TPA. During tumor promotion, mice received 2 μmol of UA and 2 μmol of Res 15 min prior to each TPA application. For the combination, Res was given 30 min and UA was given 15 min prior to each 6.8 nmol dose of TPA. TPA was administered twice-weekly for the duration of the experiment (i.e., 23 weeks). Body weight, tumor incidence (percentage of mice with papillomas)

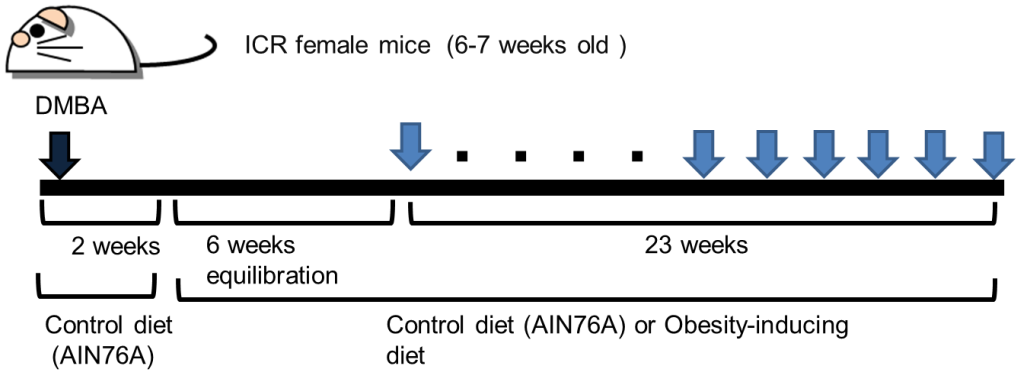
and tumor multiplicity (average number of papillomas per mouse) were measured once a week for the duration of the experiment. In addition, the surface area of all detectable papillomas was measured by digital calipers at the termination of the experiment.

A



➔ Measure tumor incidence, multiplicity and body weight once a week for 25 weeks

B



➔ Measure tumor incidence, multiplicity and body weight once a week for 23 weeks

Figure 2-3. Two stage carcinogenesis assays

A, Two-stage skin carcinogenesis for the effect of UA and other triterpenes found in *P. frutescens* on skin tumor promotion by TPA. B, Two-stage carcinogenesis assay for the effect of UA and Res on skin tumor promotion by TPA.

Label retaining cell (LRC) assay

For these experiments, 10-day old mice were injected with BrdU [50 μg/g body weight (B.W.)] *i.p*. every 12 hrs over 2 days. Seventy days later, mice were shaved on the dorsal skin and then treated twice weekly for two weeks (4 treatments total) with 0.2 ml acetone vehicle, UA (2 μmol) or Res (2 μmol) 15 min prior to each 6.8 nmol of TPA treatment. For the combination, mice received UA (2 μmol) and Res (2 μmol) 15 min and 30 min prior to TPA treatment, respectively. Mice were sacrificed 48 hrs after the last treatment and dorsal skin samples were fixed in 10% neutral-buffered formalin for 48 hrs. The fixed dorsal skin samples were embedded in paraffin and then sectioned for staining with anti-BrdUrd antibody (Abcam). LRCs (BrdUrd positive cells) in bulge region of each hair follicle were quantitated as previously described [182].

Histological analyses

For analysis of epidermal thickness and labeling index (LI) as well as the number of infiltrated inflammatory cells, mice received short-term protocol regimen. BrdUrd (100 μg/g B.W.) in PBS was injected *i.p.* to mice 30 min prior to sacrifice. Mice were sacrificed 48 hrs after the last TPA treatment and dorsal skin was excised and fixed in 10% neutral-buffered formalin. The fixed skin samples were embedded in paraffin, sectioned (4 μm) and stained with hematoxylin & eosin (H & E), toluidine blue O, anti-BrdUrd, CD3, or CD45. Epidermal thickness and LI were determined as described previously [183]. Inflammatory cell infiltration was determined by counting the number of positive-stained cells per 200 mm^2 field as previously described [10].

Preparation of epidermal protein lysates, cytosolic fractions, nuclear fractions and RNA

Mice received pretreatment with acetone or compounds before TPA treatment according to the short-term treatment protocol. Mice were sacrificed 6 hrs after the last TPA treatment. After sacrifice, epidermal protein lysates were collected as previously described [10]. Briefly, the epidermis was removed by scraping with a razor blade and then homogenized in radioimmunoprecipitation assay (RIPA) lysis buffer (50mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1mmol/L sodium orthovanadate, 1% NP-40, phosphatase inhibitor cocktail 1 and 2, and proteinase inhibitor cocktail) on ice. Lysates were centrifuged at 14,000 rpm for 15 min at 4 ºC. Supernatants were quantitated for protein amount and then subjected to Western analyses.

For electrophoretic mobility shift assays (EMSAs), the epidermal cytosolic and nuclear fractions were isolated using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific Inc). The protein lysates and nuclear/cytosolic fractions were used immediately or stored at -80 ºC until used. Epidermal RNA samples were isolated as previously described [184, 185] and subjected for quantitative real-time PCR (qPCR) analysis.

Western blot analysis

For Western blot analysis, the protein concentration of the supernatant was measured by using the protein lowry assay kit (Thermo Scientific). Aliquots of supernatant containing 30 μg protein were boiled in sodium dodecylsulfate (SDS) sample loading buffer for 5 min before electrophoresis on 6-15 % SDS-polyacrylamide gel and then transferred to the nitrocellulose membrane. The blots

were blocked with 5% bovine serum albumin (BSA) or 5% non-fat dry milk-TBST buffer [TBS containing 0.1% Tween-20] for 1 hr at room temperature. The membranes were incubated for overnight at 4℃ with 1: 1000 dilutions of primary antibodies. Blots were washed three times with TBST at the 10 min interval followed by incubation with 1: 5000 horseradish peroxidase-conjugated secondary antibodies (rabbit or mouse) for 1 hr and washed in TBST for three times. The transferred proteins were visualized with an ECL detection kit according to the manufacturer's instructions.

Antibodies against the following proteins were used: p-NF-κB p65^{S536}, IGFR-1βR, STAT3, p-STAT3^{S727}, p-STAT3^{Y705}, p-Akt^{S308}, Akt, p-p38$^{T180/Y182}$, p38, p-JNK1/2$^{T183/Y185}$, JNK1/2, p-c-Jun^{S73}, c-Jun, Fas, PDCD4, p-AMPK-α^{T172}, AMPK-α, SirT1, p-LKB1^{S416}, p-mTOR^{S2448}, mTOR, p-rS6$^{S240/244}$, rS6, p-Ulk1^{S555}, Ulk1, ATG5, LC3IIB, beclin, and α-tubulin (Cell Signaling Technology); p-EGFR^{Y1086} (Abcam); EGFR (Millipore); p21 and lamin A/C (Santa Cruz Biotechnology); p27 (BD Biosciences); actin and twist1 (Sigma).

EMSA

EMSA was performed using a DNA-protein binding detection kit according to the manufacturer's protocol (Thermo Scientific Inc.). Briefly, AP-1 oligonucleotide probe (5'-Biotin-CGC TTG ATG ACT CAG CCG GAA-3'), NF-κB oligonucleotide probe (5'-Biotin-AGT TGA GGG GAC TTT CCC AGG C-3'), and Egr-1 oligonucleotide probe (5'-Biotin-GGA TCC AGC GGG GGC GAG CGG GGG CCA-3') were labeled with biotin at the 5' end. Ten μg of nuclear protein was incubated with either DNA oligos (NF-κB, AP-1 and Egr-1) or biotin-labeled DNA oligos using LightShift EMSA

Optimization and Control Kit (Thermo Scientific Inc). Biotin-labeled DNA-protein complexes were run on 5 % polyacrylamide gel for 1 hr at 100 V and transferred on nylon membranes for 30 min at 100 V using wet transfer modules. DNA-proteins complexes were then detected using the Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific Inc.).

qPCR analysis

qPCR analyses were performed as previously described [186]. Briefly, cDNA was generated from epidermal RNA samples in a reaction (total volume of 13 µl) containing 1 µg of RNA, 2-3 µl random primers (50 ng/µl), 1 µl 10 mM dNTP mix (Invitrogen), and RNase-free water. For primer annealing, the mixtures were heated to 65 °C for 5 min and incubated on ice for 1 min. Subsequently, 4 µl 5X First-Strand buffer (Invitrogen), 1 µl 0.1 M DTT, and 1 µl SuperScript™ III reverse transcriptase (Invitrogen) were added to the mixtures and incubated at 25 °C for 5 min. For primer extension, the mixture was incubated at 50 °C for 50 min and then at 75 °C for 15 min to inactivate. For qPCR, cDNA (150 ng) was mixed with 2X TaqMan gene expression master mix (AB Applied Biosciences), 20X primer sets (IL-1α, IL-1β, IL-22, ribosomal 18 S), and nuclease-free water in a total volume of 10 µl. For qPCR of Cox-2 mRNA, 2X iTag™ universal SYBR® green supermix (Bio-rad), 1 µM primers (Cox-2, and GAPDH) nuclease-free water were added to cDNA (150 ng). The mixtures were then subjected to qPCR using ViiA™ 7 real time instrument and analysis software.

Statistical analysis

For comparisons of quantitative protein expression, gene expression, epidermal thickness, labeling index, the number of infiltrated inflammatory cells, transcriptional activities and tumor multiplicity and tumor size, the Mann-Whitney *U* test was used. A one-tailed Fisher's exact test and Mantel-Cox test was used for comparisons of tumor incidence and tumor latency, respectively. Significance in all cases was set at $p \leq 0.05$.

Chapter 3. Evaluation of pentacyclic triterpenes found in *Perilla frutescens* for inhibition of skin tumor promotion by 12-O-tetradecanoylphorbol-13-acetate.

3.1 Introduction

According to the American Cancer Society, there will be an estimated 1,658,370 new cancer cases diagnosed and 588,430 cancer deaths in the US in 2015. In addition, cancer remains the second most common cause of death in the US, accounting for nearly 1 of every 4 deaths [1]. In the mid-1970s, Michael Sporn created the term 'chemoprevention', which is defined as the use of natural or synthetic agents to reverse, inhibit or slow the process of carcinogenesis [187]. Chemoprevention may involve interruption of the multi-stage carcinogenesis process during tumor initiation, promotion, and/or progression [6]. Various tumor models have been used to evaluate cancer preventive agents. The multi-stage skin carcinogenesis model is a well-established model of epithelial carcinogenesis with distinct and definable stages of tumor development [13, 14]. This model can be used to evaluate cancer chemopreventive agents on each individual stage of the carcinogenesis process and is particularly useful for identifying potential mechanisms of chemopreventive action.

Perilla frutescens (*P. frutescens*) belongs to the annual mint family and is an edible plant frequently used in Asian countries including Korea, Japan and China. It has a pleasant flavor and taste and is used as a food ingredient. For example, perilla leaves can be added to fish, rice, soup, and vegetables and also it can be pickled for many dishes. The perilla leaf extract contains a number of constituents that have pharmacologic activity, such as triterpenoids, rosmarinic acid, luteolin, caffeic acid, epigenin, and beta-carotene [126, 188, 189]. These compounds have various biological activities reported such as anti-oxidant, hepatroprotective, anti-obesity, and anti-allergic activity [189-194]. Recently,

emerging evidence has shown perilla extract has anti-inflammatory [126, 189, 195] and anti-cancer effects [188, 194, 196, 197].

Ursolic acid (UA) is a natural pentacyclic triterpene compound present in various edible plants including *P. fructescens* [128, 129, 136, 137, 154]. It has potent cancer chemopreventive activity and possesses a wide range of pharmacological activities. UA is widely studied for its apoptotic, anti-inflammatory, and anti-tumorigenic properties, including the ability to inhibit skin tumor promotion in the mouse skin model [107, 108, 126, 127, 129]. A number of mechanisms have been attributed to the ability of UA to inhibit tumor development in these various model systems. UA has been shown to suppress multiple cell signaling pathways including, growth factor receptor activation (e.g., EGFR), and signaling through IKK/NF-κB, Akt/mTOR, Cox-2, STAT3, MMP9, and VEGF [129, 130, 137]. In addition, UA has been shown to alter levels of Bax and caspases and increase the activation of tumor suppressor proteins such as p53 and AMPK [137, 198].

In addition to UA, a number of other pentacylic triterpenes including, oleanolic acid (OA), corosolic acid (CA), 3-epi-corosolic acid (3-epiCA), maslinic acid (MA), 3-epi-maslinic acid (3-epiMA), tormentic acid (TA), pomolic aicd (PA), hyptadienic acid (HA), and augustic acid (AA) are found in *P. frutescens* [126]. These compounds are triterpenoid carboxylic acids with molecular formula $C_{30}H_{48}$ having six isoprene units and are synthesized in *P. frutescens* by cyclization of squalene. Banno *et al*., have reported that all of these compounds exhibited anti-inflammatory effects against TPA-induced ear edema [126]. They also have shown that UA, CA, 3-epiCA, TA, and 3-epiMA have potent inhibitory effects on TPA-induced Epstein-Barr virus early antigen activation [126]. Of the compounds found in *P. frutescens*, only TA, OA and UA, have previously been evaluated

for inhibition of skin tumor promotion by TPA [107, 108, 126, 127].

In the current study, we examined the effect of 7 different triterpenes including, UA, OA, AA, CA, 3-epiCA, MA, and 3-epiMA present in *P. frutescens* on epidermal proliferation, skin inflammation, inflammatory gene expression and epidermal signaling pathways induced by TPA. Six of these compounds were also evaluated for their ability to inhibit skin tumor promotion by TPA. Several of the compounds, especially 3-epiCA and MA, were found to be more effective for inhibition of skin tumor promotion than UA and are considered excellent candidates for further study of their chemopreventive effectiveness in other cancer models.

3.2 Results

3.2.1 Effect of pentacyclic triterpenes found in *P. frutescens* on skin tumor promotion by TPA.

To evaluate the anti-tumor promoting effect of UA and related triterpenes (i.e., OA, CA, 3-epiCA, MA, and 3-epiMA) present in *P. frutescens,* a two-stage skin carcinogenesis experiment was conducted using female ICR mice. AA was not included in the tumor experiment due to insufficient amount of this compound for evaluation. The control group in this experiment that was initiated with DMBA and promoted with TPA had 10.57 papillomas per mouse (see Figure 3-1A and Table 3-1). All of the pentacylic triterpenes evaluated in this experiment significantly inhibited skin tumor promotion by TPA. Pretreatment with UA resulted in a 42% inhibition of papilloma formation (6.17 papillomas per mouse; $p<0.05$, Mann-Whitney *U* test) (Table 3-1). OA (35% inhibition; 6.87 papillomas per mouse) and 3-epiMA (37% inhibition; 6.7 papillomas per mouse) also significantly inhibited skin tumor promotion by TPA ($p<0.05$; Mann-Whitney *U* test), however, these two compounds were not more effective that UA ($p>0.05$, Mann-Whitney *U* test). Of the remaining compounds evaluated, CA inhibited TPA promotion by 49% (5.38 papillomas per mouse; $p<0.05$, Mann-Whitney *U* test) when compared to TPA-only group but this was not significantly different compared to the group pretreated with UA. On the other hand, both 3-epiCA and MA inhibited skin tumor promotion by TPA to a greater extent than UA. In this regard, mice pretreated with 3-epiCA and MA exhibited 4.33 and 3.73 papillomas per mouse, respectively giving a 59% and 65% inhibition in tumor multiplicity ($p<0.05$; Mann-Whitney *U* test compared to the TPA only group and the UA + TPA group, respectively) (see again Figure 3-1A and Table 3-1).

As shown in Figure 3-1B, the incidence of papillomas in the group treated with TPA only was 97 % at week 25 (Figure 3-1B and Table 3-1). Pretreatment with UA, OA, CA and 3-epiMA did not significantly reduce the overall incidence of papillomas compared to the TPA-only treated group (p>0.05, Fisher's exact test). However, pretreatment with 3-epiCA and MA significantly reduced the overall tumor incidence (67% and 73%, respectively) when compared to TPA only group or the UA-pretreated group (both 97% incidence). These reductions in tumor incidence with 3-epiCA and MA were statistically significant (p<0.05, Fisher's exact test).

As shown in Figure 3-1C, tumor latency was also significantly affected by pretreatment with the various triterpenes. In this regard, pretreatment with UA, OA, CA, 3-epiCA, 3epiMA and MA significantly delayed tumor development when compared to TPA-only treated group (p<0.05; Mantel-Cox test). Notably, a greater increase in tumor latency was observed in the groups of mice pretreated with 3-epiCA or MA compared to the UA-pretreated group (p<0.05; Mantel-Cox test).

In summary, all of the triterpene compounds tested in this experiment significantly inhibited skin tumor promotion by TPA. UA along with OA, CA, 3-epiCA, MA, and 3-epiMA effectively inhibited the formation of papillomas promoted by TPA. Notably, 3-epiCA and MA exhibited the greatest inhibitory effect on papilloma multiplicity, incidence, and latency and these two compounds were significantly more effective at inhibiting skin tumor promotion when compared to the UA-pretreated group. As shown in Figure 3-1D, there were no significant differences in body weight between any of the triterpene-treated groups and the TPA-only treated group (p>0.05, Mann-Whitney U test). In addition, there were no signs of epidermal toxicity in mice treated

with the triterpenes indicating that the topical dosage of triterpenes used in this experiment was safe and well-tolerated.

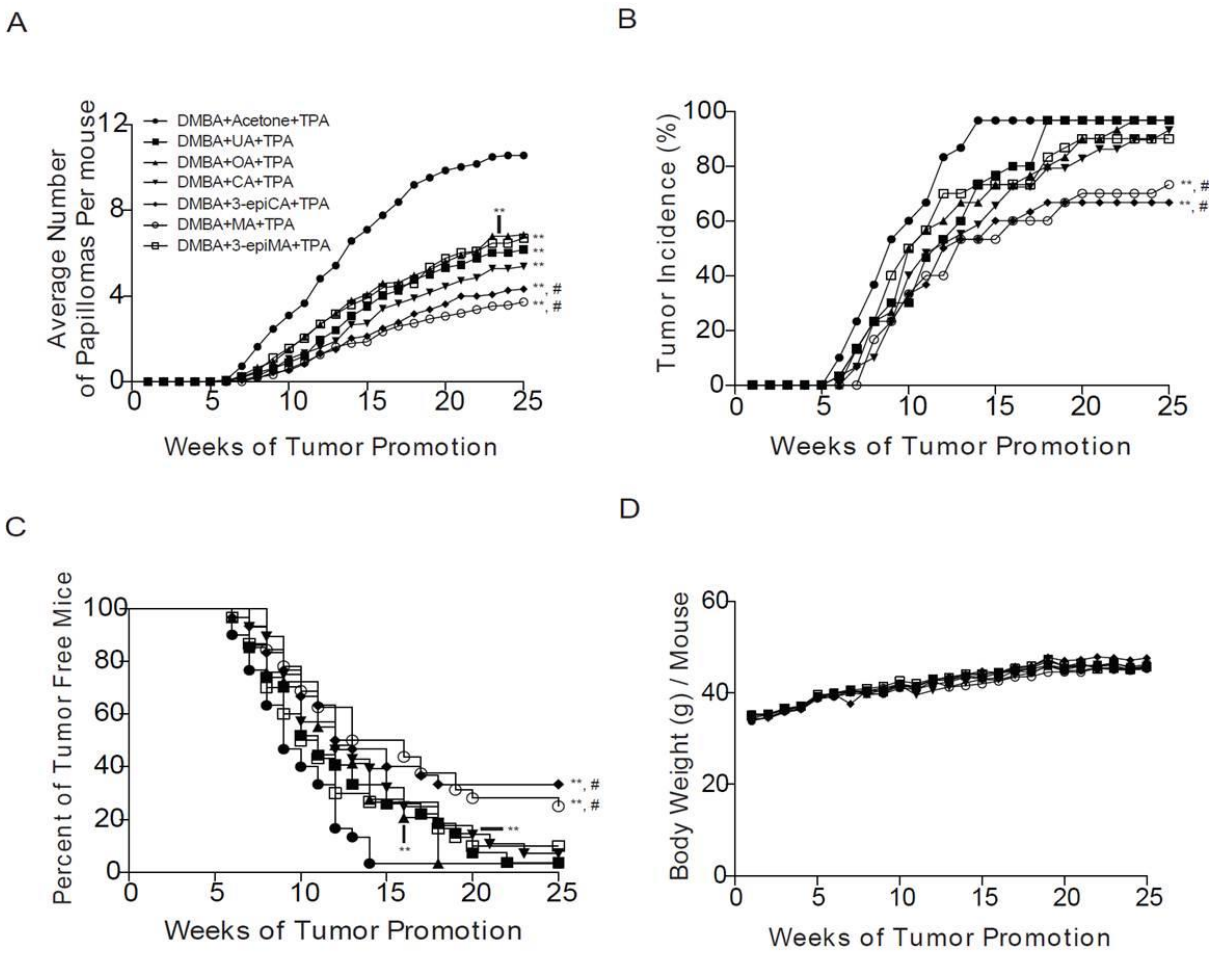


Figure 3-1: Anti-tumor skin tumor promoting effects of UA and related triterpenes found in *P. frutescens*.
Female ICR mice 7 weeks old (n=30/group) were initiated with 25 nmol DMBA. Two weeks after initiation with DMBA, mice were pretreated with either acetone vehicle (0.2 ml), UA (2 μmol), OA (2 μmol), CA (2 μmol), 3-epiCA (2 μmol), MA (2 μmol) and 3-epiMA (2 μmol) 30 min prior to each 6.8 nmol TPA treatment. All treatments were given twice-weekly. The number and incidence of papillomas as well as average body weights were measured once weekly for 25 weeks. **A.** Effect of UA and related triterpenes found in *P. frutescens* on tumor multiplicity (i.e., Y-axis on the graph shows the average number of papillomas per mouse). **B.** Effect of UA and related triterpenes found in *P. frutescens* on tumor incidence. **C.** Effect of UA and related triterpenes found in *P. frutescens* on tumor latency. **D.** Average body weight (g) per mouse. No significant difference was observed in body weight between triterpenes-untreated group and triterpenes-treated groups. **, $p \leq 0.05$ when compared to TPA-treated group; and #, $p \leq 0.05$ when compared to UA+TPA-treated group. Mann-Whitney *U* test was used for tumor multiplicity and body weight. For tumor incidence, Fisher's exact test was used. Statistical analysis of tumor latency (i.e., tumor free survival) was performed using the Mantel-Cox test.

Table 3-1. Effect of UA and related triterpenes present in *P. frutescens* on tumor multiplicity and tumor incidence.

Experimental groups	Average number of papillomas per mouse ± SEM	% inhibition	Tumor incidence %
DMBA+ Acetone + TPA	10.57 ± 1.1	-	97
DMBA+ UA + TPA	6.17 ± 0.46 **	42	97
DMBA+ OA + TPA	6.87 ± 0.62**	35	97
DMBA+ CA + TPA	5.38 ± 1.1 **	49	93
DMBA+ 3-epiCA + TPA	4.33 ± 0.8 **, #	59	67 **, #
DMBA+ MA + TPA	3.73 ± 0.6 **, #	65	73 **, #
DMBA+ 3-epiMA + TPA	6.7 ± 1.1 **	37	90

**, $p \leq 0.05$ when compared to TPA-treated group; and #, $p \leq 0.05$ when compared to UA + TPA-treated group. Mann-Whitney *U* test and Fischer's exact test were used for tumor multiplicity and tumor incidence, respectively.

3.2.2 Effect of UA and related triterpenes found in *P. frutescens* on TPA-induced epidermal hyperproliferation.

Since epidermal proliferation is important for skin tumor development during tumor promotion in the two-stage skin carcinogenesis model [13, 14], histologic analyses were conducted to determine the effects of UA and the other triterpenes on TPA-induced epidermal hyperplasia (epidermal thickness) and LI. The short-term treatment protocol was used for these experiments. As shown in Figure 3-2A and 3-2B, TPA treatment increased both epidermal thickness and LI 48 h after the last treatment when compared to vehicle (acetone)-treated group (p<0.05; Mann-Whitney *U* test). Pretreatment with all of the triterpenes reduced both epidermal thickness and LI compared to the TPA-only treated group (p<0.05, Mann-Whitney *U* test). However, 3-epiCA, MA and 3-epiMA inhibited epidermal thickness to a greater extent than UA and CA, 3-epiCA, and MA reduced the LI to a greater extent than UA (p<0.05; Mann-Whitney *U* test). Overall, these data indicate that all compounds examined in this experiment effectively inhibited TPA-induced epidermal hyperproliferation with several compounds, especially 3-epiCA and MA more effective than UA.

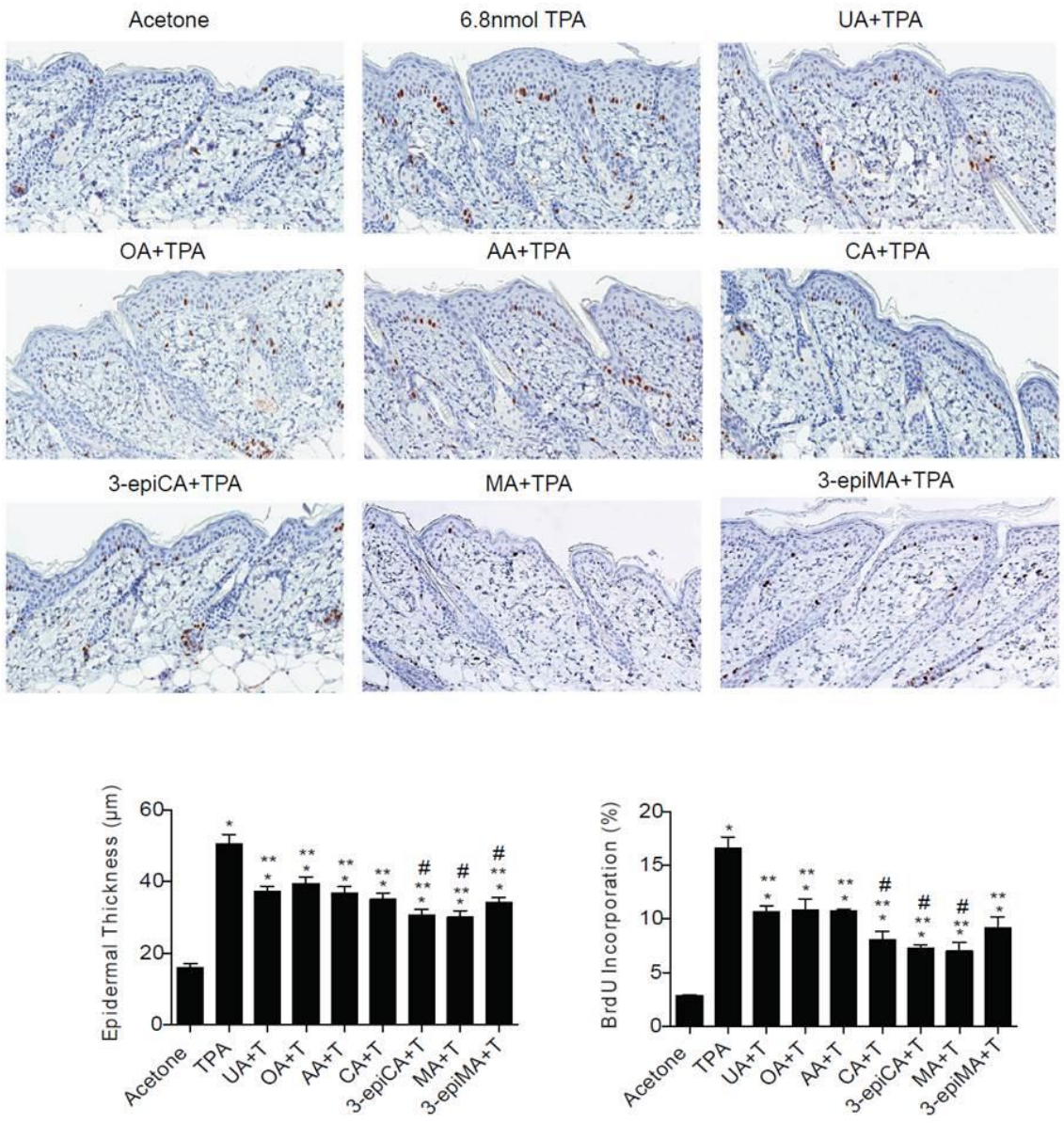


Figure 3-2: Effects of UA and related triterpenes from *P. frutescens* on TPA-induced epidermal hyperproliferation in female ICR mice.
The dorsal skin of mice (7-9 weeks of age; n=4/group) was shaved and then two days later treated with either acetone vehicle (0.2 ml), UA (2 μmol), OA (2 μmol), CA (2 μmol), 3-epiCA (2 μmol), MA (2 μmol) and 3-epiMA (2 μmol) 30 min prior to 6.8 nmol TPA. All treatments were given twice-weekly for two weeks. Forty eight hours later the last TPA treatment, dorsal skin was fixed in 10 % formalin-buffered solution, embedded in parafin and sectioned (4 μm) for BrdUrd-staining. **A.** Representative BrdU-stained skin sections (20X magnification). **B.** Quantitative analyses of the effect of UA and related triterpenes found in *P. frutescens* on TPA-induced epidermal thickness and labeling index (% of BrdU-positive cells). The graphs represent mean ± standard error of the mean (SEM). *, $p \leq 0.05$ when compared to acetone-treated group; **, $p \leq 0.05$ when compared to TPA-treated group; and #, $p \leq 0.05$ when compared to UA+TPA-treated group. Mann-Whitney *U* test was used for statistical analysis.

3.2.3 Effect of triterpenes on skin inflammation induced by TPA.

The ability of UA, OA, AA, CA, 3-epiCA, MA and 3-epiMA to inhibit TPA-induced skin inflammation was evaluated by first examining effects on the dermal infiltration of inflammatory cells (i.e., mast cells and CD3 positive T-lymphocytes). As shown in Figure 3-3A and 3-3B, all compounds significantly reduced the number of mast cells (toluidine blue O-stained cells) in the dermis in the range from 40-62% ($p<0.05$; Mann-Whitney *U* test) compared to the TPA only treated group. Again, 3-epiCA and MA were most effective at reducing the number of infiltrated mast cells and showed a significantly greater inhibitory effect compared to the UA-pretreated group ($p<0.05$, Mann-Whitney *U* test). Similar results were also observed for the number of infiltrated T-lymphocytes (CD3 positive cells) induced by TPA where all of the triterpenes significantly reduced their number compared to the TPA-only group ($p<0.05$, Mann-Whitney *U* test) (Figure 3-3B). Again, 3-epiCA and MA were the most effective compounds at inhibiting dermal T-cell infiltration induced by treatment with TPA when compared to UA ($p<0.05$; Mann-Whitney *U* test).

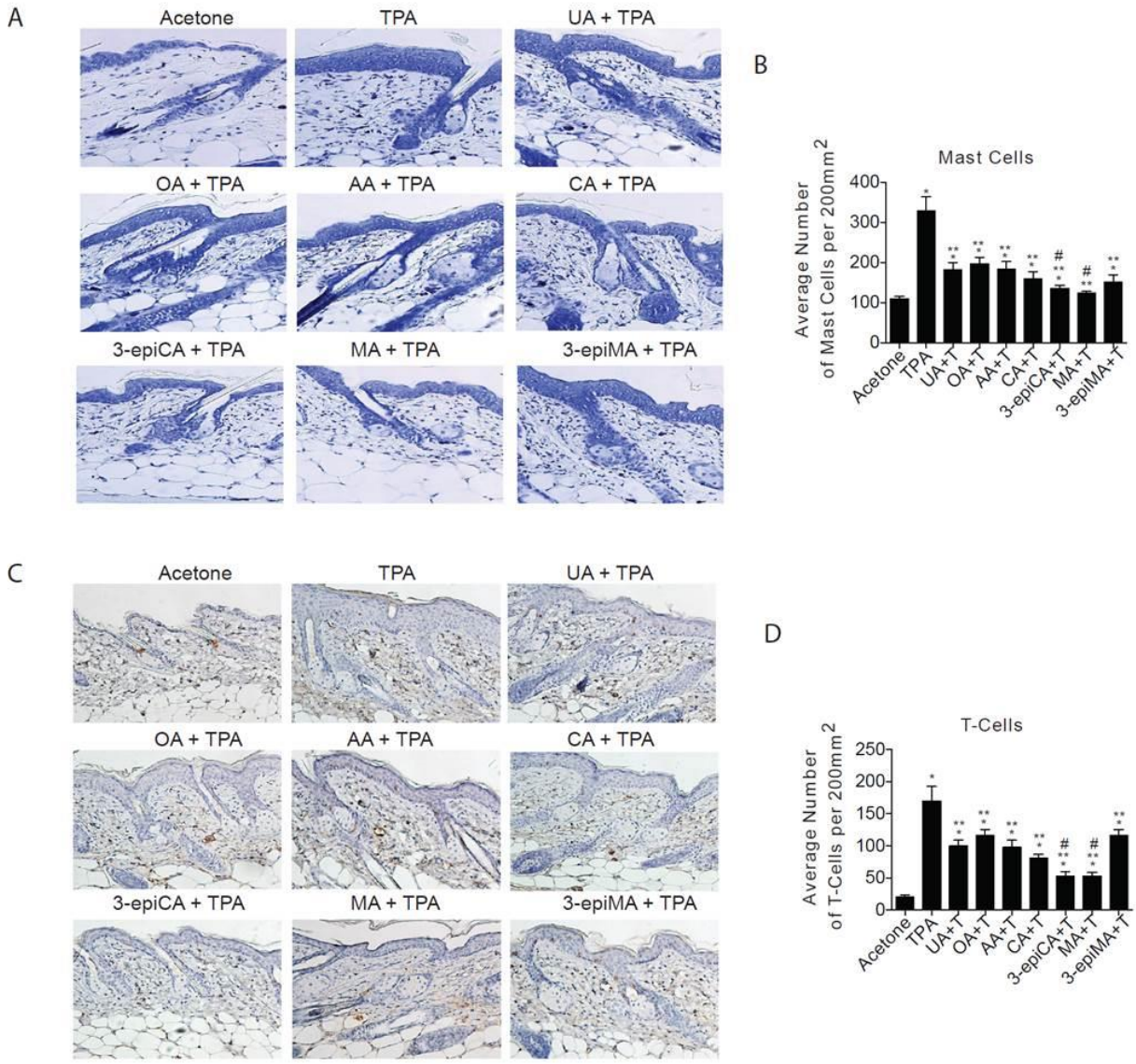


Figure 3-3: Effects of UA and related triterpenes found in *P. frutescens* on skin inflammation induced by TPA. Groups of female ICR mice (7-9 weeks of age; n=4) were shaved on the dorsal skin and two days later were treated with acetone vehicle (0.2 ml), UA (2 μmol), OA (2 μmol), CA (2 μmol), 3-epiCA (2 μmol), MA (2 μmol) and 3-epiMA (2 μmol) 30min before 6.8 nmol TPA treatment. All treatments were given twice-weekly for two weeks. Dorsal skin was collected 48 hrs after the last TPA treatment, fixed in formalin, embedded in paraffin, and 4 μm sections stained with toluidine blue O solution or CD3 antibody. **A.** Representative toluidine blue O stained skin sections. **B.** Quantitative evaluation of the effect of UA and other triterpenes found in *P. frutescens* on TPA-induced mast cells infiltration in the dermis. The graphs represent mean ± SEM. **C.** Representative histologic skin sections of $CD3^+$ staining. **D.** Quantitative evaluation of the effect of UA and other triterpenes found in *P. frutescens* on TPA-induced T-lymphocyte infiltration in dermis. *, $p \leq 0.05$ when compared to acetone-treated group; **, $p \leq 0.05$ when compared to TPA-treated group; and #, $p \leq 0.05$ when compared to UA+TPA-treated group. Mann-Whitney *U* test was used for statistical analysis.

3.2.4 Effect of triterpenes on TPA-induced inflammatory gene expression.

As shown in Figure 4, qRT-PCR analyses showed that UA and the other related triterpenes inhibited TPA-induced inflammatory gene expression in the epidermis. In this regard, mRNA levels of the following genes were evaluated: Cox-2, Il17a, Il22, Cxcl1, Cxcl2, and Vegfa. The increased expression of Cox-2 mRNA following TPA treatment was significantly reduced by pretreatment with UA, OA, CA, 3-epiCA and MA ($p<0.05$; Mann-Whitney *U* test) (Figure 3-4). MA pretreatment led to the greatest reduction in Cox-2 mRNA and this reduction was greater than that observed in the UA pretreated group ($p<0.05$; Mann-Whitney U test). Furthermore, the induction of Vegfa by TPA was lowered significantly by pretreatment with AA, CA, 3-epiCA, and MA ($p<0.05$; Mann-Whitney *U* test). mRNA levels of Il17a and Il22 was also evaluated in this experiment. TPA treatment significantly increased the expression of both Il17a and Il22 ($p<0.05$; Mann-Whitney *U* test) (Figure 3-4). Notably, the increased expression of Il17a was significantly reduced in the groups pretreated with AA, CA, 3-epiCA, and MA and a greater inhibitory effect was observed in the 3-epiCA- and MA-pretreated groups ($p<0.05$; Mann-Whitney U test) compared to the UA treated group. In addition, OA, AA, CA, 3-epiCA, and MA significantly inhibited TPA-induced Il22 expression ($p<0.05$; Mann-Whitney U test) while UA and 3-epiMA did not show a statistically significant decrease in the expression of Il22 induced by TPA. Again, 3-epiCA and MA produced the greatest inhibition of Il22 expression.

We also investigated the effects of the triterpenes on proinflammatory chemokines, Cxcl1 and Cxcl2. As shown in Figure 3-4, the expression Cxcl1 and Cxcl2 was significantly increased by TPA treatment ($p<0.05$; Mann-Whitney *U* test). Pretreatment with CA, 3-epiCA, and MA inhibited Cxcl1 mRNA induction. In addition,

all of the compounds significantly inhibited the expression of Cxcl2 by TPA ($p<0.05$; Mann-Whitney *U* test). Notably, 3-epiCA and MA, were most effective and produced greater inhibition than UA on Cxcl2 mRNA expression ($p<0.05$, Mann-Whitney *U* test).

Collectively, these data demonstrate that UA and related triterpenes found in *P. frutescens* inhibited inflammatory gene expression in the epidermis although some of these genes were differentially affected by the individual triterpenes. Notably, when looking at all the genes examined, 3-epiCA and MA produced the most significant effects on the largest number of inflammatory genes induced by TPA.

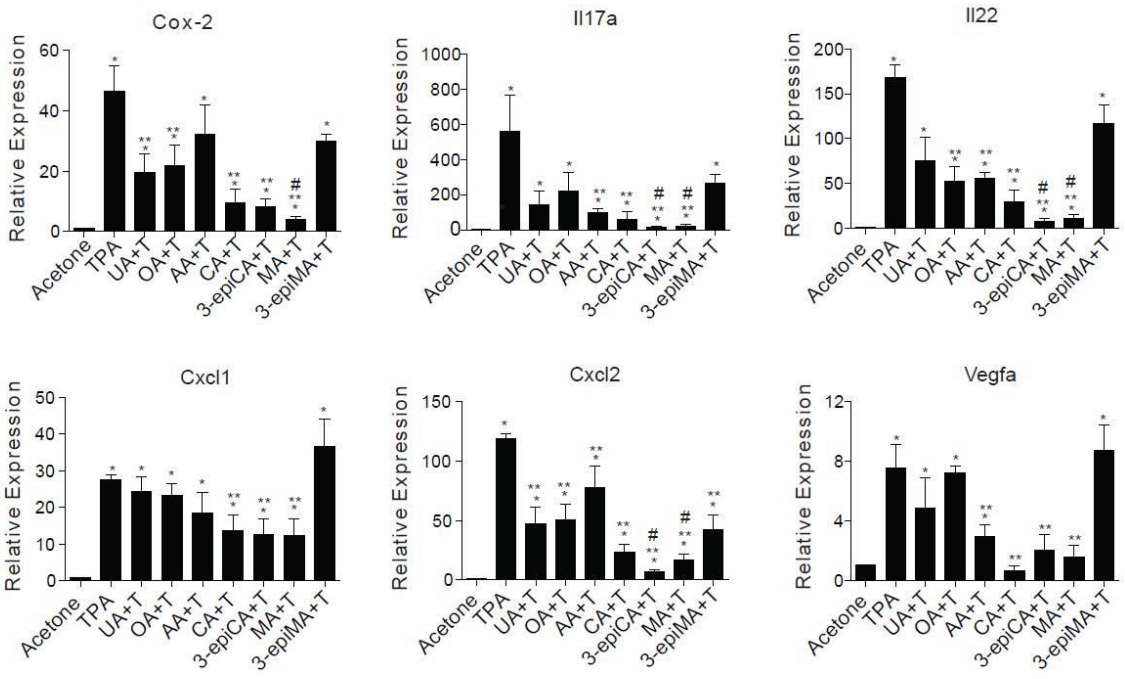


Figure 3-4: Effects of UA and a series of related triterpenes found in *P. frutescens* on TPA-induced inflammatory gene expression. Female ICR mice (7-9 weeks of age; n=4/group) were shaved on the dorsal skin and the two days later pretreated with acetone vehicle (0.2 ml), UA (2 μmol), OA (2 μmol), CA (2 μmol), 3-epiCA (2 μmol), MA (2 μmol) and 3-epiMA (2 μmol) before TPA treatment. All treatments were given twice-weekly for two weeks. Mice were sacrificed 6 hrs after the last TPA treatment and epidermal RNA was isolated to be subjected to qRT-PCR analysis. mRNA levels of Cox-2, Il17a, Il22, Cxcl1, Cxcl2, and Vegfa were normalized to Gapdh. The graphs represent mean ± SEM. *, $p \leq 0.05$ when compared to acetone-treated group; **, $p \leq 0.05$ when compared to TPA-treated group; and #, $p \leq 0.05$ when compared to UA+TPA-treated group. Mann-Whitney *U* test was used for statistical analysis.

3.2.5 Effect of triterepenes on epidermal signaling pathways induced by TPA

The effects of topical pretreatment with UA OA, AA, CA, 3-epiCA, MA and 3-epiMA on multiple signaling pathways induced by TPA in the epidermis were examined (see Figure 3-5, panels A and B). As shown in Figure 3-5A and 3-5B, groups pretreated with UA, OA, AA, CA, or 3-epiMA showed a slight inhibition of phosphorylation of IGF-1βR$^{Y1135/1136}$ (not statistically significant). However, a statistically significant inhibition was observed in the groups pretreated with 3-epiCA and MA. The inhibitory effect of 3-epiCA on activation of IGF-1βR$^{Y1135/1136}$ by TPA treatment was also significantly greater when compared to the UA-pretreated group (p<0.05; Mann-Whitney *U* test). On the other hand, none of the triterpene compounds showed inhibition of TPA-induced p-EGFR^{Y1086} levels. Evaluation of the effect of the triterpenes on p-Src^{Y416} levels was also examined. Significant inhibition of the phosphorylation of p-Src^{Y416} was observed in groups pretreated with CA, 3-epiCA, MA and 3-epiMA (p<0.05; Mann-Whitney *U* test). Again, similar to the result of IGF-1βR signaling, 3-epiCA was more effective at reducing p-Src^{Y416} levels than UA following treatment with TPA (p<0.05; Mann-Whitney *U* test).

The inhibitory effect of the triterpenes was also examined on STAT3 phosphorylation (both the Y705 and the S727 sites) induced by TPA (Figure 3-5A and 3-5B). As shown in the figure, CA, 3-epiCA, MA and 3-epiMA significantly decreased the phosphorylation of STAT3 at S727 (p<0.05; Mann-Whitney *U* test) while rest of the compounds tested did not show a statistically significant reduction in p-STAT3^{S727} level when compared to the TPA-only treated group. Notably, 3-epiCA, MA, and 3-epiMA showed the greatest inhibitory effect (p<0.05; Mann-Whitney *U* test). In addition, 3-epiCA and MA significantly inhibited the phosphorylation of p-STAT3^{Y705}

(p<0.05; Mann-Whitney U test) while the other compounds did not significantly alter STAT3 phosphorylation at this site. A downstream target of STAT3, Twist1, was also examined. Pretreatment with CA, 3-epiCA, MA, and 3-epiMA reduced the level of Twist1 induced by TPA (p<0.05; Mann-Whitney U test).

Additional analyses were performed to examine the effect of the triterpenes on other signaling pathways such as Cox-2, JNK1/2, c-Jun, Pdcd4 and p27. As shown in Figure 3-5A and 5B, the level of Cox-2 protein was significantly reduced by all of the compounds except AA and the reduction by 3-epiCA and MA was greater than that observed in the UA-pretreated group (p<0.05; Mann-Whitney U test). In addition, all triterpenes excluding OA significantly reduced the phosphorylation of JNK1/2$^{T183/Y185}$ that was stimulated by TPA treatment (p<0.05; Mann-Whitney U test) and AA, CA, 3-epiCA, MA and 3-epiMA had a significant inhibitory effect on TPA-induced c-Jun phosphorylation at S73. Notably, a greater reduction in p-c-Jun^{S73} was observed in the groups pretreated with CA, 3-epiCA, and 3-epiMA (p<0.05; Mann-Whitney U test) compared with the UA-pretreated group. The effect of the pentacyclic triterpenes on tumor suppressor proteins, Pdcd4 and p27, was investigated. The decreased level of Pdcd4 after TPA treatment was partially reversed by CA, 3-epiCA, MA and 3-epiMA (p<0.05; Mann-Whitney U test) whereas none of compounds had significant effects on p27 levels following treatment with TPA.

Several studies including those from our group have reported AMPK activators (e.g. Metformin, AICAR, compound C) inhibit skin cancer *in vitro* and *in vivo* [11, 105, 199], therefore the effect of the triterpenes on AMPK signaling following TPA treatment was also evaluated. As shown in Figure 3-5A and 5B, TPA treatment induced a

modest activation of AMPK (assessed by phosphorylation at T172). Pretreatment with UA did not significantly increase AMPK activation while pretreatment with the other compounds tested significantly increased the phosphorylation of AMPK-α^{T172} above that observed following TPA treatment (p<0.05; Mann-Whitney *U* test). The status of SirT1 and LKB1 was also evaluated. As shown in Figure 3-5A and 3-5B, activation of LKB1 (phosphorylation at S428) was enhanced by pretreatment with CA, 3-epiCA, MA and 3-epiMA (p<0.05; Mann-Whitney *U* test) while all triterpene compounds except UA significantly increased the level of SirT1 (p<0.05, Mann-Whitney *U* test). As expected, phosphorylation of the downstream target of AMPK, Ulk1 at S555 was also elevated by pretreatment with AA, CA, 3-epiCA, MA and 3-epiMA. Notably, the dramatic upregulation as seen in AMPK signaling after 3-epiMA pretreatment was also observed in the phosphorylation of Ulk1 at S555 after pretreatment with this compound.

In summary, the data in Figure 3-5 demonstrate that pretreatment with the various triterpenes that were evaluated caused alterations in a number of signaling pathways important for skin tumor promotion by TPA. Several of the compounds, including 3-epiCA and MA showed greater effects on a broad range of signaling pathways that may have contributed to their greater ability to inhibit skin tumor promotion by TPA.

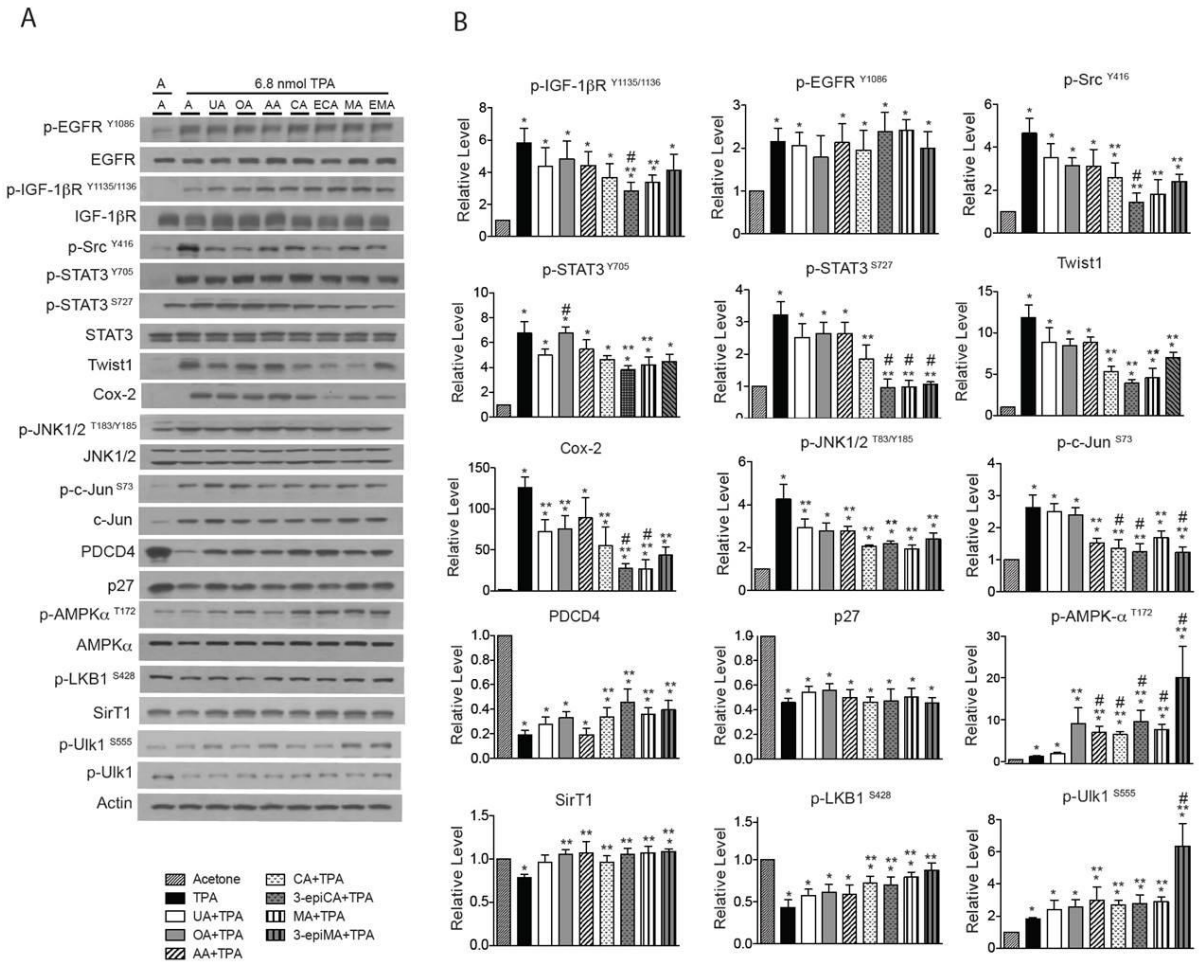


Figure 3-5: Effects of UA and related triterpenes from *P. frutescens* on TPA-induced epidermal signaling pathways in female ICR female. Groups of mice (n=4/group) were shaved on the dorsal skin and the two days later treated with acetone vehicle, UA (2 μmol), OA (2 μmol), CA (2 μmol), 3-epiCA (ECA, 2 μmol), MA (2 μmol) and 3-epiMA (EMA, 2 μmol) 30 min prior to 6.8 nmol TPA treatment. All treatments were given twice-weekly for two weeks. Six hrs after the last TPA treatment, epidermal lysates were prepared for Western blot analysis. **A.** Representative Western blots for the effect UA and related triterpenes on TPA-induced signaling pathways. **B.** Quantitative evaluation of the Western blot analysis. Phosho-proteins are normalized to both total protein and actin. The levels of twist1, Cox-2, Pdcd4, p27 and SirT1 were normalized to actin. The graphs represent mean ± SEM of three independent experiments. *, $p \leq 0.05$ when compared to acetone-treated group; **, $p \leq 0.05$ when compared to TPA-treated group; and #, $p \leq 0.05$ when compared to UA+TPA-treated group. Mann-Whitney *U* test was used for statistical analysis.

3.3 Discussion

In current study, all of the triterpenes tested significantly inhibited skin tumor promotion by TPA. 3-EpiCA and MA were the most effective compounds at inhibiting the development of papillomas and were more effective than the prototype and more widely studied UA. Mechanistic studies revealed that all of the tripterpenes tested had the ability to inhibit TPA-induced epidermal hyperproliferation and skin inflammation. Again, the overall inhibitory effects on epidermal proliferation and skin inflammation were the greatest in the groups pretreated with 3-epiCA or MA and significantly greater than seen in the UA +TPA group. Analysis of epidermal signaling pathways induced by TPA revealed that there were variable effects of the different compounds on individual signaling pathways. However, the greater ability of 3-epiCA and MA to inhibit skin tumor promotion was associated with greater reduction of Cox-2 and Twist1 proteins and inhibition of activation (i.e., phosphorylation) of IGF-1R, Stat3 and Src. Collectively, the current data demonstrate that triterpenes from *P. frutescens* inhibit skin tumor promotion by inhibiting signaling pathways associated with epidermal proliferation and inflammation.

Several of the pentacyclic triterpenes found in *P. frutescens* have been widely studied. In this regard, UA has been shown to have apoptotic, anti-inflammatory, and anti-tumorigenic effects [130, 135-137]. UA and OA are often found together in plants, including *P. frutescens* and possess similar pharmacological properties [129, 135-137]. OA, like UA, also exhibits anti-inflammatory, gastro-protective, wound-healing, and anti-microbial propoerties [138, 139]. In addition, several studies have shown its anti-cancer effects in both *in vitro* and *in vivo* studies [127, 140]. OA inhibits proliferation and induces apoptosis in many cancer cell lines including breast, lung,

and skin [141-144]. CA also exists in abundance in the plant kingdom, including bananas and loquat as well as *P. frutescens* [126, 200]. A number of studies have shown that CA has anti-diabetic, anti-oxidant, anti-inflammatory, apoptotic, and anti-cancer activities. Its anti-cancer effects have not been as extensively studied *in vivo* although several *in vitro* studies have demonstrated growth inhibitory effects in cancer cell lines including gastric, colon, cervix, leukemia and lung [157-163]. MA, also known as crategolic acid, has been isolated from *P. frutescens* as well as other edible plants such as olive fruit, spinach, eggplant, mustard, basil and legumes [148]. Recently, the anti-proliferative or apoptotic properties of MA have been demonstrated in various cancer cell lines such as colon, liver, bladder, uterus and breast [149-154].

As noted in the Introduction, of the compounds evaluated in the current study, only UA and OA have previously been shown to inhibit skin tumor promotion by TPA [107, 108, 127, 128]. In a recent study by our group, topical pretreatment with 2 μmol of UA reduced the number of TPA promoted skin papillomas by 39% [108]. In the current study, the same dose of UA given before TPA reduced papilloma formation by 42 %. Thus, these two independent studies show that UA when given topically to the same mice (female ICR) and under similar experimental conditions produced essentially the same level of inhibition of tumor promotion. Tokuda *et al*. reported that topical application of OA inhibited skin tumor promotion by TPA in female ICR mice using considerably lower doses [127]. In this study, they also compared the anti-tumor promoting effect of OA with UA using two different treatment protocols. Both OA and UA inhibited skin tumor promotion by TPA to a similar extent when the compounds were given prior to each TPA application over a 20 week period. Surprisingly, both compounds also were shown to inhibit skin tumor development

when given only once prior to the first TPA application. The mechanisms for the observed inhibition of skin tumor promotion by both OA and UA in either protocol were not reported in this earlier study. In the current study, UA and OA given at the same topical dose produced a very similar inhibition of skin tumor promotion (42% vs 35%, respectively; Table 3-1). The potential anti-cancer effects of CA and MA have been reported in a number of *in vitro* studies, however, only a few studies have evaluated their inhibitory effects in cancer models. Li *et al.* reported that subcutaneous administration of MA (10 and 50 mg/kg) inhibited growth of pancreatic cancer cells in a xenograft mouse model [155]. In another study, MA was given in the diet (100 mg/kg) to APC Min/+ mice and was shown to reduce colon carcinogenesis by 45 % [156]. In addition, MA inhibited both the size and weight of bladder tumors in a xenograft mouse model [152]. Recently, Yoo *et al*. have reported that CA possess suppressive effects on angiogenesis and lymphangiogenesis *in vivo* using a CT-26 colon carcinoma animal model [164].

In contrast to UA and OA, no studies to date have reported on the anti- skin tumor promoting effects of CA, 3-epiCA, MA and 3-epiMA. Therefore, our results report for the first time the ability of these triterpenoids that are found together in *P. frutescens* to inhibit skin tumor promotion by TPA in the two-stage skin carcinogenesis model. As shown in Figure 3-1 and Table 3-1, all 4 of these compounds had the ability to inhibit skin tumor promotion by TPA when given topically 30 min prior to each TPA application. This was seen primarily in the ability to inhibit the formation of papillomas that were promoted by TPA. Significant effects were also observed on tumor latency. When compared to the activity of UA for inhibition of skin tumor promotion, two compounds (i.e., 3-epiCA and MA) exhibited greater ability to inhibit

skin tumor promotion as assessed by effects on tumor multiplicity, tumor incidence, and tumor free survival (i.e., latency) (see again Figure 3-1 and Table 3-1).

In our previous study, topical application of 2 μmol UA prior to TPA application was shown to significantly inhibit Cox-2, NF-κB p65^{S536} and Akt^{T308} signaling as well as reductions in activation of Src^{Y416}, STAT3^{Y705}, and JNK1/2$^{T183/Y185}$ [108]. In the current study, the effect of 2 μmol of UA, OA, AA, CA, 3-epiCA, MA and 3-epiMA were compared for their effects on some of these same pathways as well as others (Figure 3-5). All compounds reduced Cox-2 expression and the levels of p-JNK1/2 although the reduction in Cox-2 level with AA pretreatment was not statistically significant. In addition, all of the compounds reduced the levels of p-c-Src^{Y416} although the values with UA, OA and AA were not statistically significant. Some of the triterpenes, notably, 3-epiCA and MA produced a greater degree and a broader range of inhibition of signaling pathways. Further analyses revealed that these two compounds, in addition to inhibiting Cox-2, p-JNK1/2$^{T183/Y185}$ and p-Src^{Y416}, significantly reduced p-IGF-1R$^{Y1135/1136}$, p-Stat3^{S727}, Twist1, p-c-Jun^{S73} and increased Pdcd4, p-AMPK-α^{T172}, Sirt1, p-LKB1^{S428} and p-ULK1^{S555}. Thus, the greater inhibitory activity of these two compounds was associated with a greater ability to modulate multiple growth factor and inflammatory signaling pathways in the epidermis.

During tumor promotion stage, repeated topical treatments of TPA produce and maintain chronic epidermal cell proliferation [13]. Initiated cells have a selective growth advantage during TPA induced epidermal hyperplasia proliferation and

undergo clonal expansion to form pre-malignant papillomas [13, 14, 21]. Therefore, we examined the anti-proliferative effect of the triterpenes on TPA-induced epidermal hyperproliferation. Our previous data indicated that topical treatment of UA inhibited both epidermal thickness and LI induced by TPA [108]. In the current study, UA and the other related triterpenes significantly inhibited TPA-induced epidermal thickness and LI. However, 3-epiCA and MA, again showed the greatest effect on epidermal hyperproliferation and these two compounds were more effective than the group pretreated with UA.

Another important aspect of tumor promotion is chronic inflammation. Upregulation and secretion of pro-inflammatory molecules by TPA treatment recruits inflammatory cells such as mast cells, monocytes, leukocytes, T-and B-lymphocytes,, and macrophages into the dermis [22, 23]. The number of these cells is increased in the dermis adjacent to the epidermis and they promote tumor growth by producing growth factors, cytokines, and chemokines [22, 23]. Data previously published from our group suggested that multiple applications of TPA increased the number of dermal infiltrated inflammatory cells such as mast cells, T-lymphocytes, and macrophages in mouse skin [10, 108]. Topical treatment with UA and other chemopreventive agents such as rapamycin and resveratrol, have been shown to decrease the number of these cells in the dermis following TPA treatment [10, 108]. In addition, Banno *et al.* demonstrated the anti-inflammatory effect of UA and some other triterpenes including OA, AA, CA, 3-epiCA, CA, MA and 3-epiMA on TPA-induced ear edema [126, 201]. In this study, they determined ID_{50} values of triterpenoids and the range of ID_{50} on TPA-induced inflammation in mouse ear was from 0.09 to 0.15 mg/ear showing that no dramatic difference of ID_{50} was observed

among the groups pretreated with UA, AA, CA, 3-epiCA, MA or 3-epiMA dissolved in MeOH-$CHCl_3$-H_2O vehicle. However, given the higher ID_{50} value (0.3 mg/ear) of OA, OA was less effective than the others. As shown in Figure 3-3, UA and related triterpenes significantly decreased the number of dermal mast cells and T-lymphocytes induced by TPA. Again, the most effective compounds were 3-epiCA and MA. In addition, these two compounds were the most effective overall at inhibiting TPA-induced inflammatory gene expression (Figure 3-4)

In conclusion, evaluation of a series of pentacyclic triterpenes found in *P. frutescens* showed that all of these compounds inhibited skin tumor promotion by TPA and that 3-epiCA and MA were more effective than UA. The inhibitory effects of all compounds and the greater effect of several compounds such as 3-epiCA and MA on skin tumor promotion were due to the reduction of epidermal hyperproliferation, skin inflammation, and alterations in a number of epidermal signaling pathways critical for tumor promotion. Although some of these compounds were previously shown to have anti skin tumor promoting activity (UA and OA), this is the first comprehensive comparison of a series of pentacyclic triterpenes found in *P. frutescens* for their effects on skin tumor promotion *in vivo* and included new compounds not previously evaluated (i.e., CA, 3-epiCA MA and 3-epiMA) . Furthermore, we found that two of these compounds were more active than UA and have provided a potential mechanistic basis for this increased activity. Overall, these compounds and especially 3-epiCA and MA, deserve further evaluation for their cancer chemopreventive activity.

Chapter 4. Effect of the combination of UA and Res on skin tumor promotion by TPA[1]

4.1 Introduction

UA is a natural pentacyclic triterpenoid carboxylic acid found in many plants including *P. fructescen* (Japanese basil), rosemary, apples, elder flowers and many others. UA has been shown to have apoptotic, anti-inflammatory and anti-tumorigenic effects in various cancer models including prostate, ovary, stomach, intestine, and skin [126, 127, 129]. Further studies have revealed that UA has broad-spectrum anti-carcinogenic effects including prevention of DNA damage, inhibition of EGFR/MAPK signaling, inhibition of angiogenesis, activation of apoptotic pathways, and inhibition of Akt/mTOR, NF-κB, Cox-2, and STAT3 signaling pathways [129, 130]. Although several studies have reported that UA inhibited carcinogen and 12-O-tetracanoylphorbol-13-acetate (TPA)-induced inflammation, hyperplasia and tumor promotion in mouse skin [107, 126, 127], its inhibitory mechanism on skin tumor promotion is not fully understood. Recently, several studies reported that UA has an anti-obesity effect and mimics some of the effects of calorie restriction (CR) by modulating Akt/mTOR signaling pathways [131-133]. UA has also been shown to activate the LKB1/AMPK pathway for inhibition of adipogenesis [134].

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1. Cho J, Rho O, Junco JJ, Carbajal S, Siegel D, Slaga TJ and DiGiovanni J. Effect of Combined Treatment with Ursolic Acid and Resveratrol on Skin Tumor Promotion by 12-O-tetradecanoylphorbol-13-acetate. Cancer prevention research. 2015

Development of methodology: J. Cho, O. Rho, J. DiGiovanni
Acquisition of data (provided animals, acquired and managed patients,provided facilities, etc.): J. Cho, J.J. Junco, D. Siegel, J. DiGiovanni
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Res is a phytoalexin and is present in grapes, berries, peanuts and red wine. Res has been shown to have cardiovascular benefit and anti-diabetic effects in both mice and humans. In addition, Res was shown to inhibit skin tumor promotion and also inhibit the growth of many cancer cell lines, including breast, prostate, colon and liver [107, 167, 169, 172, 179]. Mechanisms associated with the anti-tumor promoting effects of Res include inhibition NF-κB, AP-1, and Cox-2 [167-169]. Several reports have suggested that Res also mimics some of the effects of CR on life span in worms and other model organisms, especially by inhibiting inflammation and mTOR signaling [170, 173]. Res also mimics effects of CR by increasing SirT1 and AMPK activation [171]. Boily *et al*. have suggested that the anti-promoting effect of Res on mouse skin is at least partially mediated by SirT1 [106].

Emerging evidence suggests that combinations of phytochemicals may be an effective strategy to achieve a greater chemopreventive effect than with single agents [174, 180, 181]. Several studies have shown that combinations of natural compounds can produce potential synergistic inhibitory effects in various cancers (e.g., Res + grape seed extract and ellagic acid + grape seed extract) [176-181]. Recently, Junco *et al*. reported that Res potentiates the growth inhibitory effect of UA in mouse skin papilloma and carcinoma cell lines [202]. Thus, combining agents may provide the most rational and effective approach to cancer chemoprevention. In addition, using combinations of phytochemicals may produce overall effects that more similarly mimic CR.

In the present study, topical treatment with a combination of UA + Res produced a greater inhibitory effect on skin tumor promotion by TPA than with either agent alone.

Further mechanistic studies revealed that this combination produced a greater inhibition of multiple growth factor and inflammation signaling pathways as well as greater upregulation of tumor suppressor genes such as p21 and PDCD4. Interestingly, the combination of UA + Res induced a dramatic increase of p-AMPK-α^{Thr172} and its downstream target p-Ulk1^{Ser555}. Collectively, the current data suggest that combined treatment of UA + Res is a more effective inhibitor of skin tumor promotion than either UA or Res given alone. The mechanism for this greater inhibition appears to be multi-faceted with similarities to changes observed with CR.

4.2 Results

4.2.1 Effect of UA + Res on skin tumor promotion by TPA.

The ability of a combination of UA + Res to inhibit skin tumor promotion by TPA was evaluated in ICR mice maintained on either an overweight control diet or a diet-induced obesity diet (DIO diet). After completion of the tumor experiment, the tumor responses in both diet groups were similar for all groups (see Figs. 4-1A and 1B). Therefore, the data for the corresponding treatment groups on each diet were combined as presented in Fig. 4-2. As shown in Fig. 4-2B, pretreatment with UA or Res alone inhibited tumor multiplicity by 38.6% and 20.8%, respectively. The reduction in tumor multiplicity with UA was statistically significant ($p<0.05$; Mann-Whitney *U* test) compared to the group treated with TPA alone. Pretreatment with the combination of UA + Res resulted in 56% reduction in tumor multiplicity that was significantly lower when compared to both the Res + TPA and UA + TPA groups ($p<0.05$; Mann-Whitney *U* test). Furthermore, the incidence of papillomas in the mice treated with UA + Res was significantly lower than that observed in the TPA and Res + TPA treated groups (Fig. 4-2A) ($p<0.05$; Fisher's exact test) but not the UA + TPA group. An effect on tumor latency was also observed as shown in Fig. 4-2C. In this regard, the percent of tumor-free mice treated with the combination of UA + Res was significantly higher than that of the TPA only and Res + TPA groups over the 23 week observation period ($p<0.05$; Mantel-Cox test) but not the UA + TPA treated group.

As shown in Fig. 4-2D, the combination of UA + Res significantly reduced the size of papillomas compared to the TPA only group as well as both the UA + TPA and Res + TPA groups ($p<0.05$; Mann-Whitney *U* test). Thus, the combination was more effective at reducing both the number and size of papillomas when compared to

either agent given alone.

Body weight gain for the 23 week experiment for each group is shown in Fig. 4-1C and 1D. As expected, there were significant differences in body weight between untreated mice on the overweight control and DIO diets (41.57 g ± 2.49 and 56.57 g ± 2.39, respectively; $p < 0.05$; Mann-Whitney *U* test). No significant differences were observed in body weight between the treated groups in mice on either the control diet or DIO diet. Overall, these data suggest that the combined treatment of UA + Res, at the doses used, had a greater inhibitory effect on skin tumor promotion compared to the groups pretreated with either of the compounds alone and with no apparent toxicity.

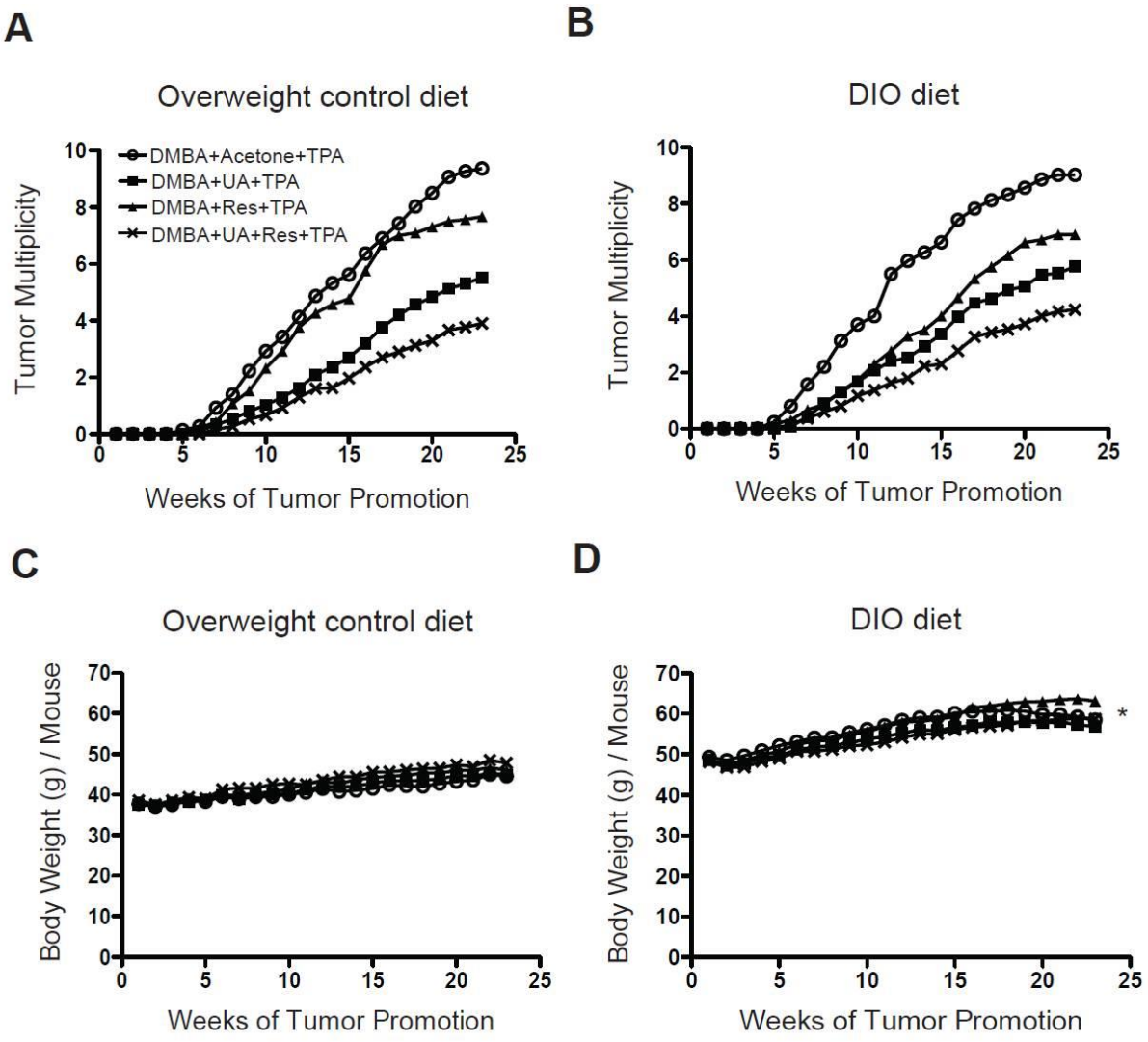

Figure 4-1. Effect of UA + Res on skin tumor promotion in female ICR mice on the overweight control and DIO diet (Panels A and B, respectively) and on weight gain (Panels C and D, respectively). Panel A, tumor multiplicity in mice maintained on the overweight control diet. Panel B, tumor multiplicity in mice on the DIO diet. Panel C, average body weight of groups of mice on the control diet. Panel D, average body weight of groups of mice on the DIO diet. All groups on the DIO diet had body weights significantly greater than those on the control diet (*, $p<0.05$; Mann-Whitney *U* test). No differences in body weight were observed between treatment groups in mice on either the control or DIO diet.

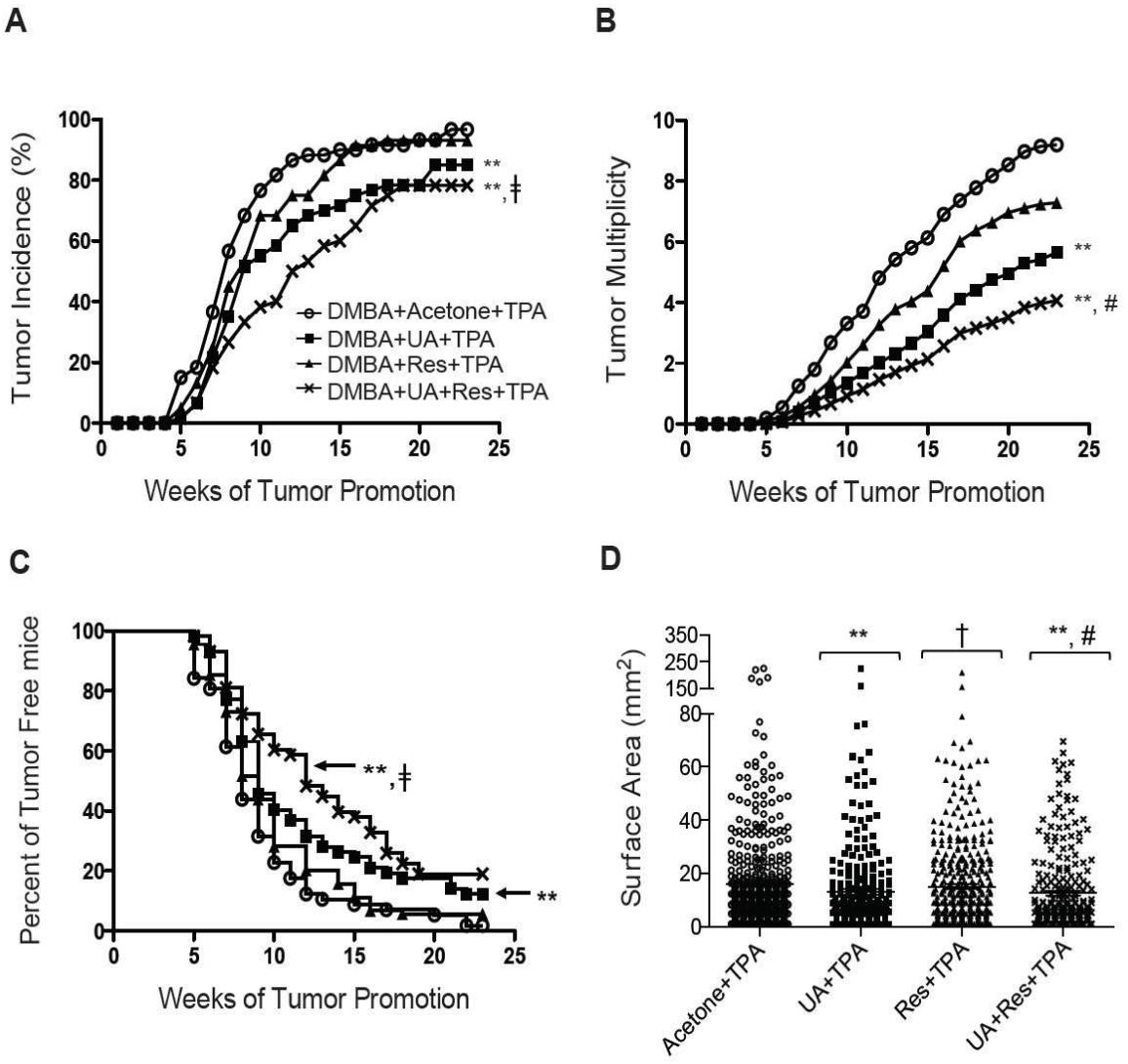

Figure 4-2. Effect of UA + Res on skin tumor promotion in ICR mice. Panel A, Incidence of tumors (percentage of mice with papillomas). The percentage of mice with papillomas in the group treated with UA + Res +TPA was significantly lower than TPA (**) or Res + TPA (‡) treated groups (p<0.05; Fisher's exact test). Panel B, tumor multiplicity (average number of papillomas per mouse). Both the UA and the UA + Res pretreated groups had significantly reduced tumor multiplicity compared to the TPA-treated group (**, p< 0.05). The tumor multiplicity in the UA + Res + TPA-treated (#, p<0.05; Mann-Whitney U test) was also significantly lower than both UA + TPA and Res + TPA-treated group. Panel C. tumor latency (tumor free survival). Significant differences were observed between the UA + TPA and UA + Res + TPA compared to the TPA only group (**, p<0.05; Mantel-Cox test). Percent of tumor free mice in the combination group (‡, p<0.05; Mantel-Cox test) was greater than the Res + TPA group. Panel D, tumor size. The surface area of papillomas was measured at the 23rd week. **, p<0.05 when compared to TPA group; †, p<0.05 when compared to UA + TPA group; and #, p<0.05 when compared to both the Res + TPA and UA + TPA groups. The Mann-Whitney U test was used for all statistical comparisons of tumor size.

4.2.2 Effect of UA + Res Treatment on TPA-induced epidermal hyperproliferation

As shown in Figs. 4-3A and 4-3B, UA or Res alone significantly reduced BrdU incorporation and epidermal thickness following TPA treatment ($p<0.05$; Mann-Whitney *U* test). However, treatment with the combination of UA + Res produced a greater inhibition of BrdU incorporation and epidermal thickness induced by TPA compared to that observed with either of the compounds given alone with TPA ($p<0.05$; Mann-Whitney *U* test).

As shown in Fig. 4-4, LRCs in acetone-treated mice were confined to the hair follicle bulge-region as expected based on previous studies [182, 185]. However, after a two-week treatment regimen with TPA, the LRCs can be seen moving up and out of the hair follicle into the interfollicular epidermis (Fig. 4-4A). Pretreatment with UA or Res partially inhibited the effect of TPA on proliferation and migration of LRCs. However, treatment with the combination of UA + Res prior to application of TPA produced a greater inhibitory effect on the proliferation and migration of these cells compared to the UA or Res only treated groups (Fig. 4-4B; $p<0.05$; Mann-Whitney *U* test).

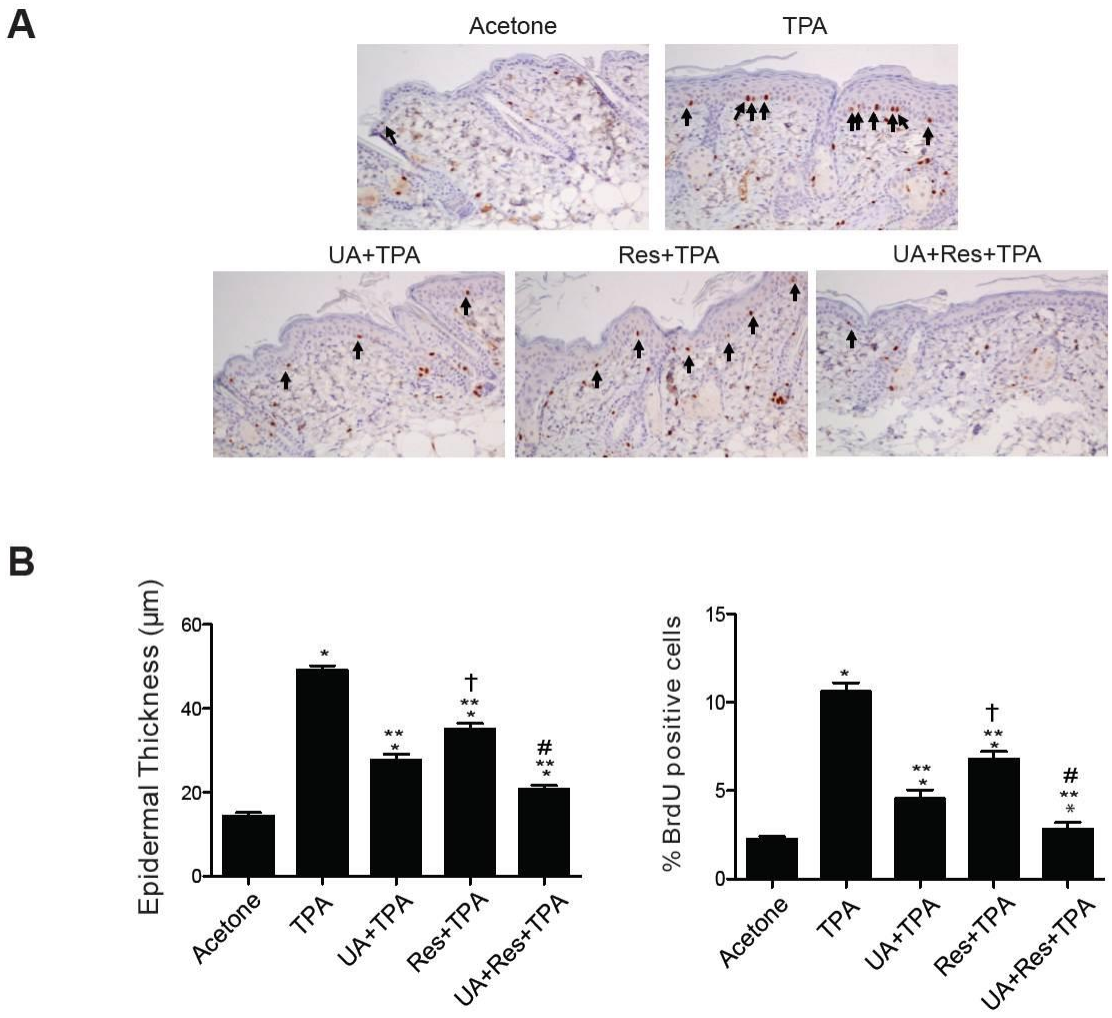

Figure 4-3. Effect of UA + Res on TPA-induced epidermal hyperproliferation in ICR mice. Female ICR mice at 7-8 weeks of age maintained on standard chow diet were treated topically with the short-term protocol. Dorsal skin was collected at 48 hrs after the last treatment for histological evaluation. Sections were stained with H&E and for BrdU incorporation. Panel A, representative sections of BrdU stained skin. Arrows indicate BrdU-positive cells. Magnification, X 20 Panel B, quantitative analysis of the effects of UA, Res or UA + Res on TPA-induced epidermal thickness and labeling index (% BrdU positive cells). The values in panel B represent the means ± SEM. *, $p<0.05$ when compared to the acetone treated group; **, $p<0.05$ when compared to the TPA treated group; †, $p<0.05$ when compared to the UA + TPA group; #, $p<0.05$ when compared to UA + TPA and Res + TPA group. All statistical analyses were performed using the Mann-Whitney *U* test.

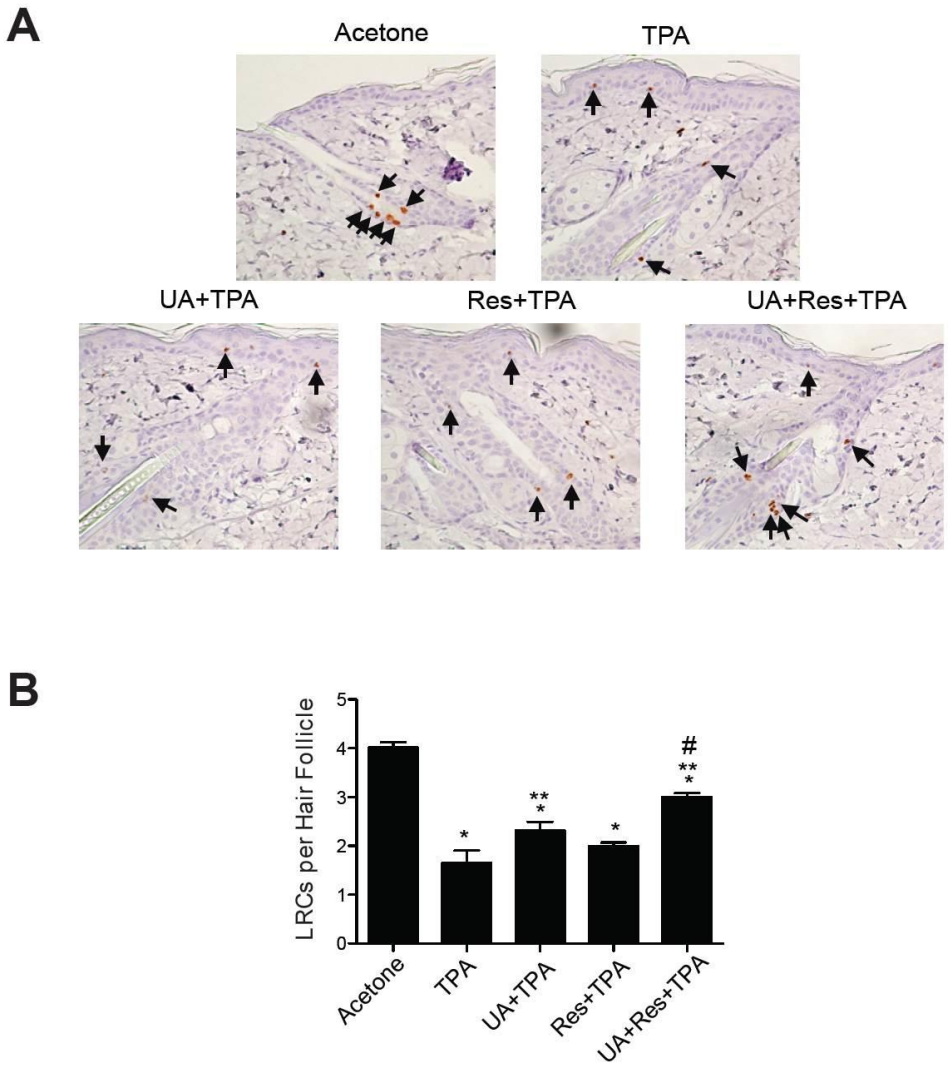

Figure 4-4. **Impact of UA + Res on the proliferation/migration of hair follicle stem/progenitor cells after TPA treatment in female FVB mice.** Mice at 10-days of age were injected with BrdU (50 µg/g B.W) every 12 hrs over two days and then 70 days later mice were treated topically using the multiple treatment protocol with acetone vehicle, TPA (6.8 nmol), UA (2 µmol) + TPA, Res (2 µmol) + TPA or UA (2 µmol) + Res (2 µmol) + TPA. Dorsal skin sections were collected and subjected to BrdU staining for the presence of labeling-retaining cells (LRCs) as described in Materials and Methods. Panel A, representative sections of BrdU-stained skin. Arrows indicate the positions of BrdU-labeled cells in the various skin sections. Magnification, X 20 Panel B, quantitative evaluation of the effect of UA, Res, or UA + Res on TPA-induced proliferation/migration of hair follicle stem/progenitor cells. LRCs in the hair follicle and epidermis were counted and quantitated as previously described [182]. *, $p<0.05$ when compared to acetone group; **, $p<0.05$ when compared to TPA group; and #, $p<0.05$ when compared to UA + TPA and Res + TPA group. The Mann-Whitney *U* test was used for all statistical comparisons.

4.2.3 Effect of UA + Res on TPA-induced epidermal signaling pathways.

As shown in the Fig. 4-5A and 5B, UA + Res significantly inhibited TPA-activated p-STAT3^{Tyr705}, p-Akt^{Thr308}, p-NF-κB p65^{Ser536}, p-JNK1/2$^{Thr183/Tyr185}$, p-c-Jun^{Ser73}, and p-p38 MAPK$^{Thr180/Tyr182}$, whereas UA or Res alone either produced no significant effects or a moderate inhibition of phosphorylation of these proteins relative to the combination. Cox-2 induction by TPA was not significantly decreased by pretreatment with either UA or Res alone, however, the combination of UA + Res produced a statistically significant inhibition of Cox-2 induction by TPA. The levels of several tumor suppressors were also evaluated (see again Fig. 4-5A and 5B). The combination of UA + Res significantly reversed the effect of TPA on PDCD4 and p21 levels while pretreatment with either compound alone had no effect. In contrast, none of the treatments reversed the effects of TPA treatment on p27 levels.
The phosphorylation of both EGFR^{Tyr1086} and Src^{Tyr416} was also significantly inhibited by the combination of UA + Res at the doses and time points examined whereas neither UA nor Res alone significantly inhibited phosphorylation of these proteins. On the other hand, the increased level of Fas induced by TPA was decreased by treatment with Res alone and the combination of UA + Res but not with UA. Again, the combination was the most effective at inhibiting the increase in Fas seen following treatment with TPA.

As shown in Fig. 4-6A and 6B, treatment with TPA alone produced a ~2.5-fold increase in p-AMPK-α^{Thr172} compared to the acetone treated control group. Both UA and Res when given with TPA further increased p-AMPK-α^{Thr172}, while the combination of UA + Res together with TPA produced an even greater activation of AMPK-α that was significantly greater than with either UA or Res given alone

(p<0.05). The level of SirT1 was not changed by treatment with TPA or pretreatment with any of the compounds given together with TPA, including the combination of UA + Res (again see Fig. 4-6A and 6B). TPA treatment reduced the level of p-LKB1^{Ser428} compared to the acetone group, however, neither UA nor Res had any further effect. In contrast, the level of p-LKB1^{Ser428} was further decreased when the combination of UA + Res was given before TPA treatment. TPA treatment led to activation of mTORC1 signaling as previously reported [10, 182, 203], however, the levels of p-mTORC1^{Ser2448} and its downstream target, p-S6-ribosomal protein$^{Ser240/244}$, were not affected by treatment with UA, Res or the combination of UA + Res. Notably, the level of p-Ulk1^{Ser555} was significantly increased when the combination of UA + Res was given together with TPA. The combination of UA + Res when given together with TPA significantly increased the level of LC3IIB compared to the acetone, TPA and UA + TPA groups while the levels of ATG5 and Beclin1 that were reduced by TPA treatment were not significantly altered further by any of the treatments.

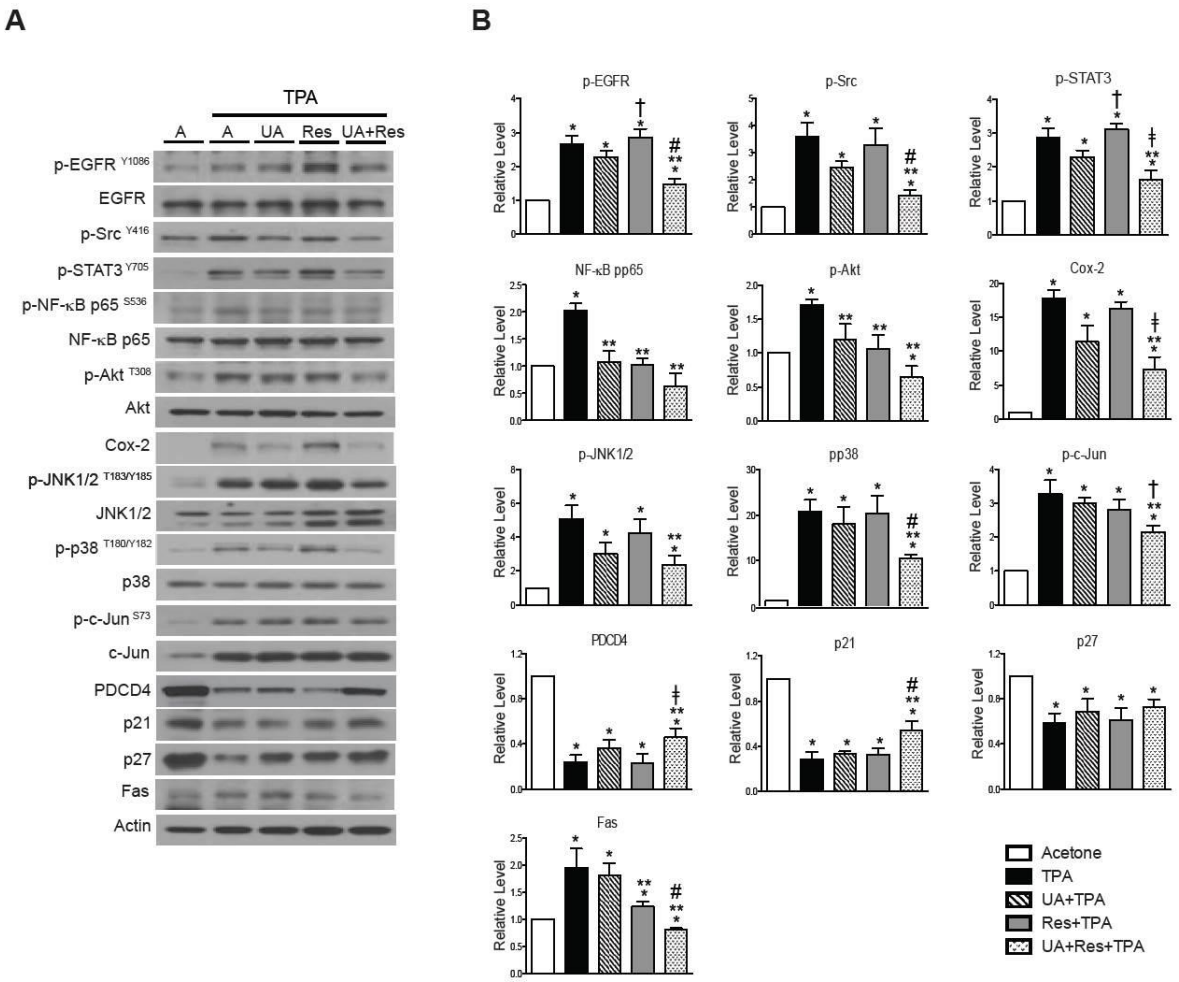

Figure 4-5. Effect of UA + Res on TPA-induced signaling pathways in epidermis of female ICR mice. Western blot analyses were performed using pooled epidermal protein lysates from mice (n=4-5/group) receiving treatment with the short-term protocol. Panel A, representative Western blot analyses of multiple signaling pathways. Panel B, quantitative evaluation of Western blot data. Values represent means ± SEM from at least 3 independent experiments. *, $p<0.05$ when compared to the acetone treated group; **, $p<0.05$ when compared to the TPA treated group; †, $p<0.05$ when compared to the UA + TPA treated group; ‡, $p<0.05$ when compared to the Res + TPA treated group; and #, $p<0.05$ when compared to the UA + TPA and Res + TPA treated groups. The Mann-Whitney *U* test was used for all statistical comparisons.

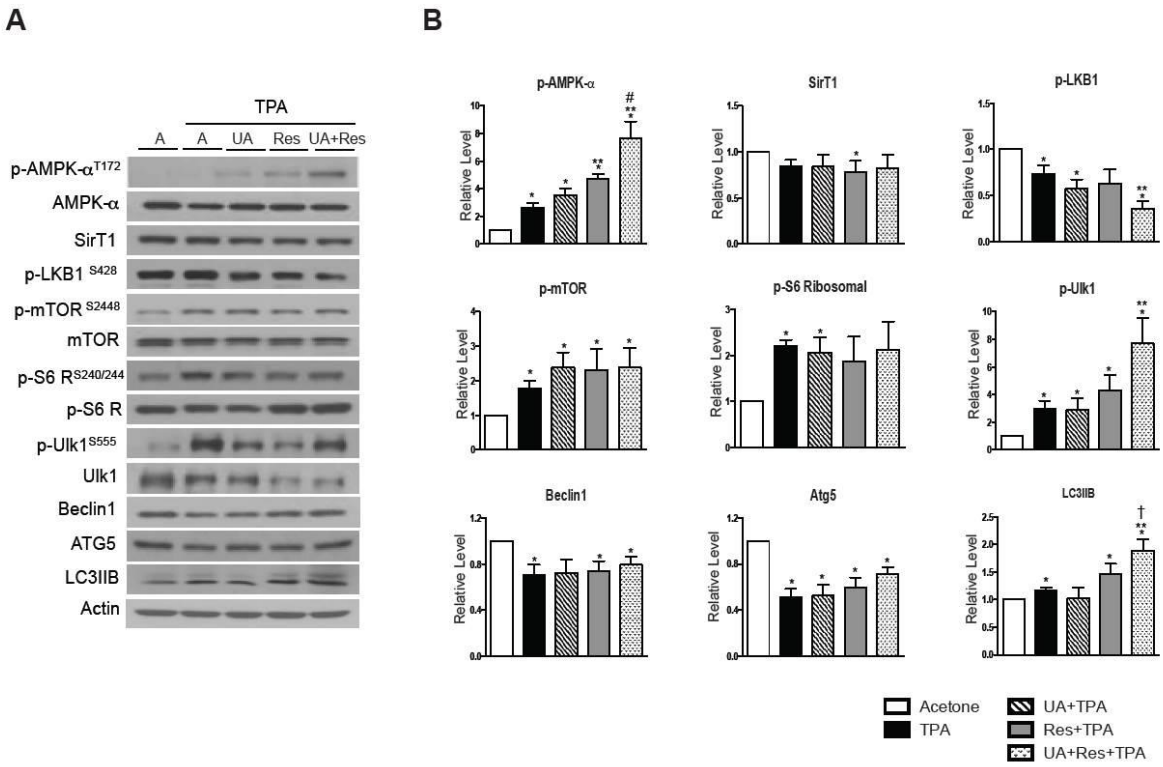

Figure 4-6. Effect of UA + Res on TPA-induced AMPK and mTORC1 signaling pathways in epidermis of female ICR mice. Western blot analyses were performed using pooled epidermal protein lysates from mice (n=4-5/group) that received multiple treatments with short-term protocol. Panel A, representative Western blot analyses. Panel B, quantitative evaluation of the effect of UA, Res or UA + Res on TPA-induced AMPK-α signaling pathway. Values represent means ± SEM from at least 3 independent experiments. *, $p<0.05$ when compared to acetone group; **, $p<0.05$ when compared to TPA group; †, $p<0.05$ when compared to UA + TPA group; ‡, $p<0.05$ when compared to the Res + TPA treated group; and #, $p<0.05$ when compared to UA + TPA and Res + TPA group. The Mann-Whitney *U* test was used for statistical comparisons.

4.2.4 Effect of UA + Res on TPA-induced inflammation and inflammatory gene expression.

As shown in Fig. 4-7A, the levels of IL-1α, IL-1β, IL-22 and Cox-2 mRNA were increased following treatment with TPA (given twice weekly for two weeks) and significantly decreased in the UA + Res pretreated group (p<0.05; Mann-Whitney U test). With the exception of UA pretreatment on Cox-2 mRNA, neither UA nor Res pretreatment significantly reduced the mRNA levels of these inflammatory genes. As shown in Figs. 4-7B pretreatment with UA and Res decreased the number of mast cells in the dermis seen following TPA treatment, however, an additional decrease in the number of dermal mast cells was observed after treatment with UA + Res + TPA (p<0.05; Mann-Whitney U test). UA alone and UA + Res also produced a significant decrease in the number of $CD45^+$ cells in dermis (p<0.05) (Fig. 4-7C). Again, the combination of UA + Res produced the greatest reduction in the numbers of both mast cells and $CD45^+$ cells. Representative stained sections for mast cells and $CD45^+$ cells are shown in Fig. 4-8, panels A and B, respectively.

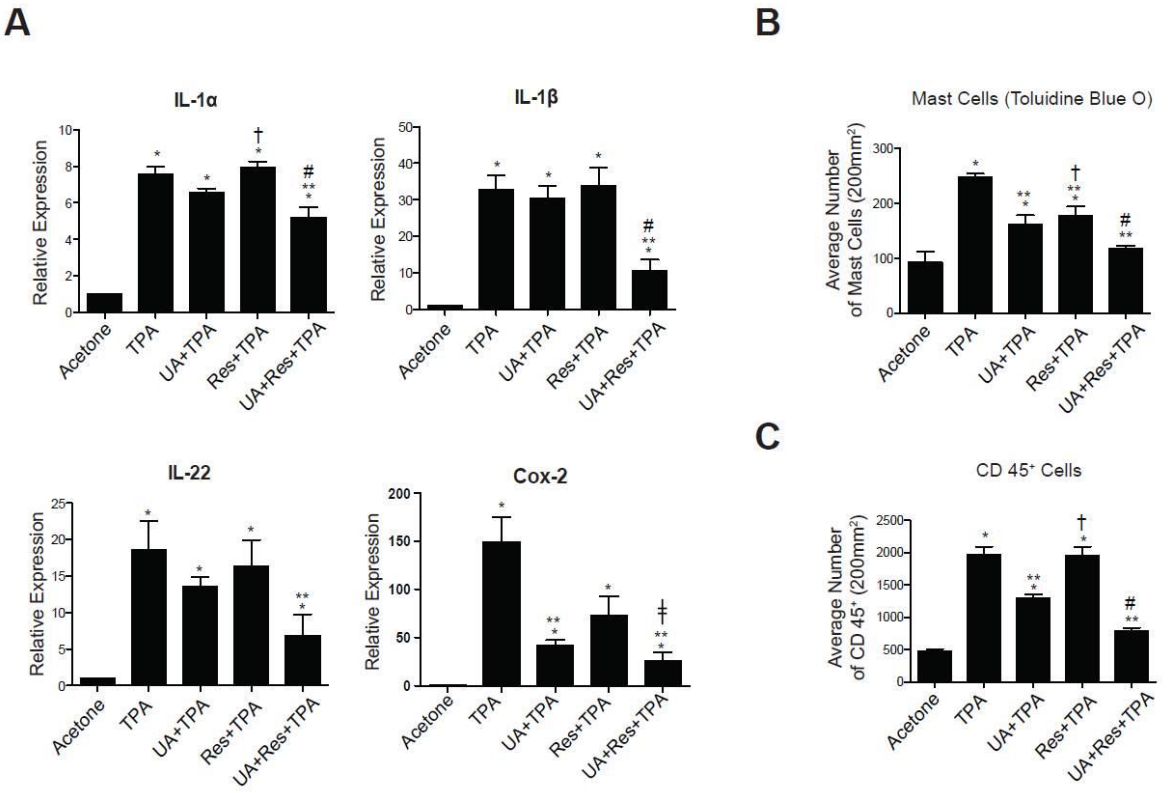

Figure 4-7. **Effect of UA + Res on TPA-induced inflammatory gene expression and inflammatory cell infiltration**. Epidermal RNA samples were prepared from groups of female ICR mice (n=4-5/group) treated using the short-term protocol. RNA samples were then subjected to qRT-PCR analysis as described in Materials and Methods. Panel A, qPCR analysis of IL-1α, IL-1β, IL-22, and Cox-2. mRNA levels of IL-1α, IL-1β, and IL-22 were normalized to 18S and the mRNA level of Cox-2 was normalized to GAPDH. Panel B, quantitative evaluation of the effect of UA, Res and UA + Res on the number of mast cells in the dermis 48 hrs after the last TPA treatment. Positive cells were counted per 200 mm^2. Panel C, quantitative analysis of the effect of UA, Res and UA + Res on the number of CD45 positive cells in the dermis 48 hrs after the last TPA treat. Positive cells were counted per 200 mm^2. The graphs in all cases represent means ± SEM of at least 3 independent experiments. *, p<0.05 when compared to acetone group; **, p<0.05 when compared to TPA group; †, p<0.05 when compared to UA + TPA group; ‡, p<0.05 when compared to Res + TPA group; and #, p<0.05 when compared to UA + TPA and Res + TPA group. The Mann-Whitney *U* test was used for statistical comparisons.

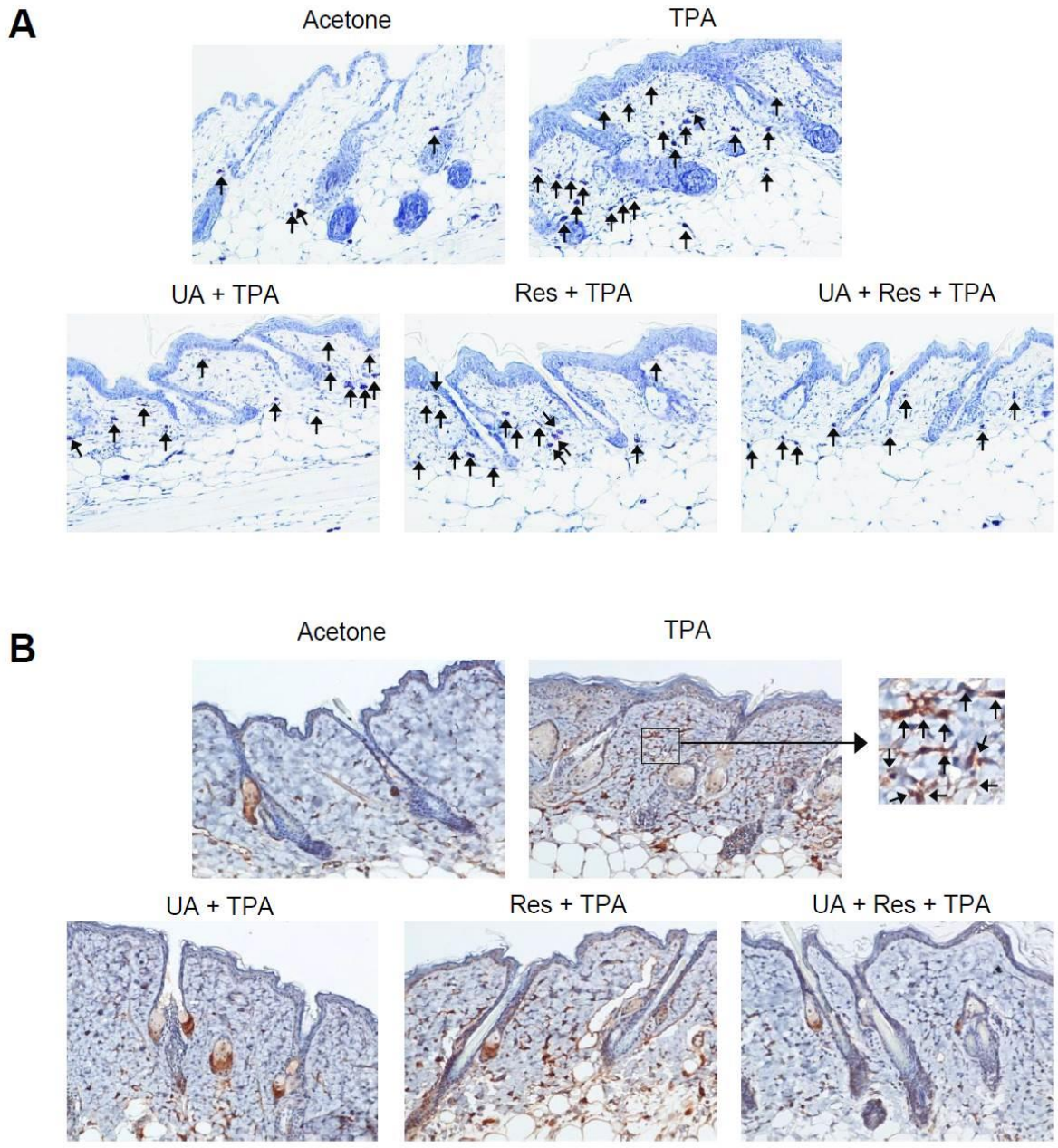

Figure 4-8. Effect of UA + Res on TPA-induced dermal infiltration of inflammatory cells. Panel A, representative sections of toluidine blue O stained skin. Panel B, representative sections of $CD45^{+}$ stained skin. Arrows in each panel indicate the corresponding stained cells. Magnification, X 20

4.2.5 Effect of UA + Res on NF-κB, Egr-1, and AP-1 DNA binding activities induced by TPA.

Treatment with TPA significantly increased the amount of NF-κB, Egr-1 and AP-1 bound to their consensus DNA binding oligos (Figs. 4-9A-C and Figs 4-10A-C). The TPA-induced increase in DNA binding activity of all three transcription factors was significantly reduced by pretreatment with the combination of UA + Res. Pretreatment with UA or Res alone significantly reduced binding of NF-κB and UA pretreatment significantly reduced binding of AP-1. Thus, the combination of UA + Res was highly effective at inhibiting the activation of all three of these transcription factors by TPA. Additionally, the nuclear translocation of NF-κB, Egr-1 and AP-1 induced by TPA was also significantly inhibited by UA + Res to a greater extent than pretreatment with either UA or Res (Figs. 4-9G-I and Figs. 4-10D-F).

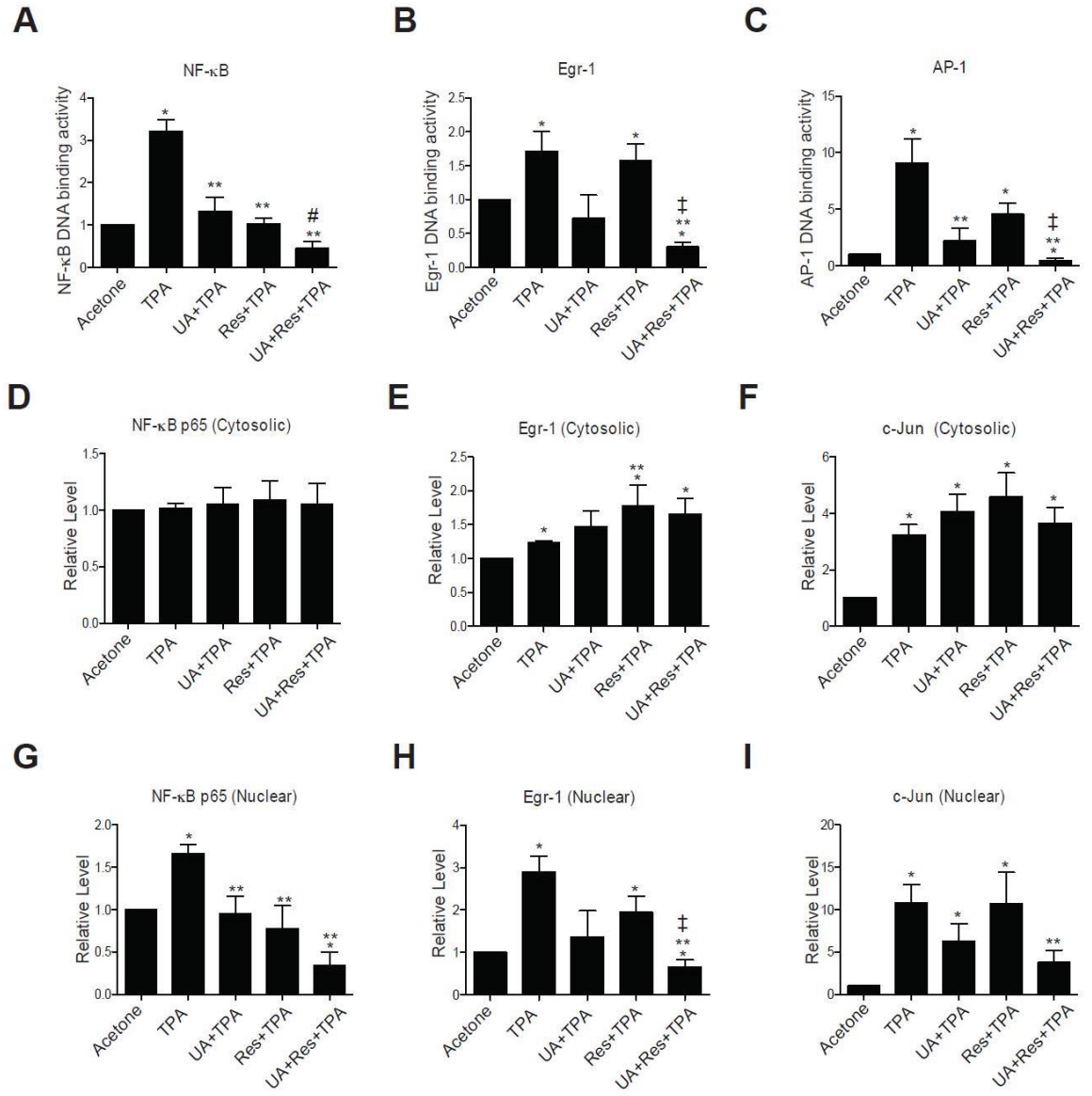

Figure 4-9. Effect of UA + Res on DNA binding activity and nuclear translocation of NF-κB, Egr-1, and AP-1 induced by TPA. Groups of female ICR mice (4-5 mice per group) on the control diet were treated with the short-term protocol. Pooled epidermal nuclear and cytosolic fractions were obtained. The nuclear fractions were then subjected to EMSA and Western blot analyses. Western blot analyses were also performed with the epidermal cytosolic fractions. Panels A-C, quantitative analysis of EMSA for NF-κB, Egr-1 and AP-1, respectively. Panels D-F, quantitative evaluation of Western blot data for cytosolic levels of NF-κB, Egr-1, and AP-1, respectively. Panels G,-I, quantitative analysis of Western blot data for nuclear translocation of transcription factors NF-κB p65, Egr-1, and AP-1, respectively. The graphs represent mean values ± SEM of at least 3 independent experiments. *, $p<0.05$ when compared to acetone group; **, $p<0.05$ when compared to TPA group; ‡, $p<0.05$ when compared to Res + TPA group; and #, $p<0.05$ when compared to UA + TPA and Res + TPA group. The Mann-Whitney *U* test was used for statistical comparisons.

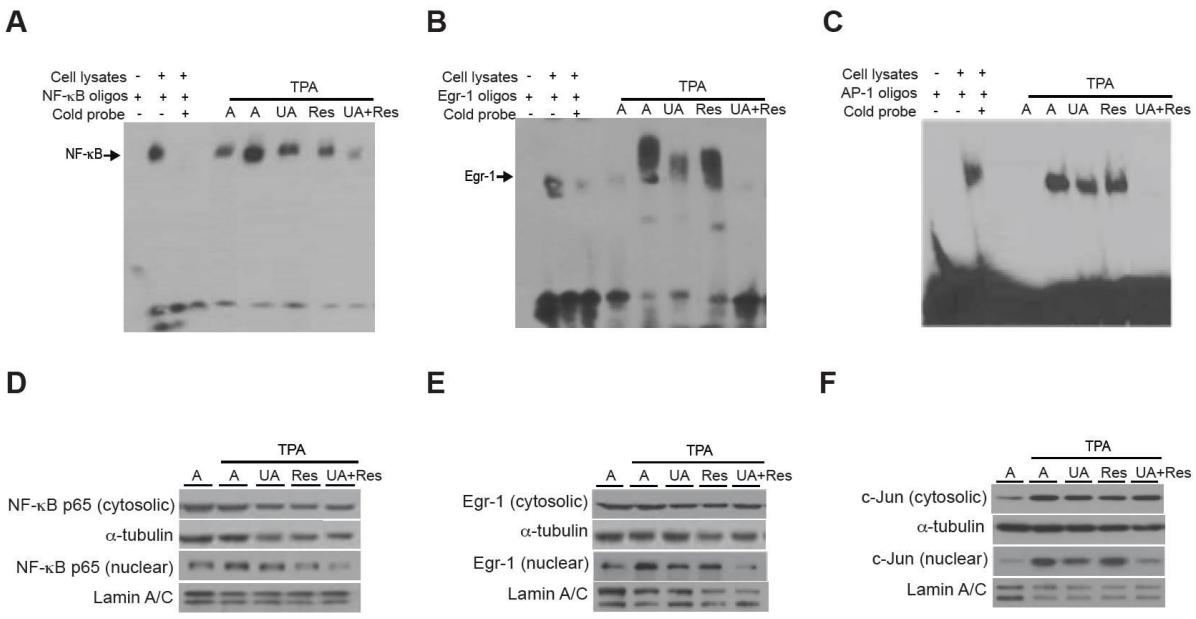

Figure 4-10. **Representative blots showing the effect of UA + Res on DNA binding activity and nuclear translocation of NF-κB, Egr-1, and AP-1 induced by TPA.** Panels A-C, representative EMSA blots for NF-κB, Egr-1, and AP-1, respectively. Panels D-F, representative Western blot analyses for cytosolic and nuclear levels of each transcription factor as indicated.

4.3 Discussion

In the present study, topical application of UA + Res followed by TPA treatment inhibited skin tumor promotion to a greater extent when compared to the groups treated with either Res + TPA or UA + TPA alone. Further analyses revealed that the greater ability of the combination to inhibit skin tumor promotion correlated with a greater ability to inhibit epidermal proliferation induced by TPA. In addition, combined treatment with UA + Res produced greater inhibitory effects on TPA-induced epidermal signaling pathways including EGFR, STAT3, Fas, Src, Akt, Cox-2, NF-κB, p38 MAPK, and JNK1/2. Notably, treatment with the combination also increased the levels of the tumor suppressor proteins p21 and PDCD4 compared to the groups treated with the individual compounds. Both UA and Res treatment followed by TPA increased AMPK-α activation, however, the combination of UA + Res together with TPA produced an even greater activation of AMPK-α. Further studies revealed that the activation of NF-κB, Egr-1, and AP-1 (DNA binding activity and nuclear translocation) were significantly inhibited by the combination of UA + Res compared to the groups treated with either UA or Res alone. The combination also produced greater effect on TPA-induced inflammation and inflammatory gene expression. Overall, the current data indicate that combined treatment with UA + Res led to a greater inhibitory effect on skin tumor promotion than either compound alone via effects on multiple events and pathways critical to the process of skin tumor promotion.

As noted in the Introduction, UA was previously shown to have inhibitory effects on TPA-induced skin inflammation as well as skin carcinogenesis [107, 127]. Res was

also shown to be as an effective inhibitor on skin tumor promotion [107, 167, 169, 204] along with inhibitory effects on breast, colorectal, hepatic, pancreatic, and prostate cancers [172]. Recently, several studies have shown potential combinatorial chemopreventive effects with these agents in preclinical models of cancer. For example, melatonin was shown to potentiate the inhibitory effect of UA on proliferation and apoptosis in colon cancer cells by modulating multiple signaling pathways including caspases, PARP, NF-κB and Cox-2 [205]. Kowalczyk *et al*. [107] tested a combination of 2% calcium D-glucarate (CG) given in the diet, with either 2.5 μmol of Res or 1 μmol of UA applied topically in two-stage skin carcinogenesis model. In this study, UA applied alone and in combination with CG showed inhibitory effects on skin tumor incidence and multiplicity.

Combinations of Res with other phytochemicals have been shown to have a greater inhibitory effect in several tumor models. For example, combined dietary administration of Res, quercetin and catechin (combinations at 0.5, 5 or 25 mg/kg) reduced primary tumor growth of breast cancer xenografts in a nude mouse model [206]. Res + black tea polyphenol inhibited mouse skin tumor growth by modulating MAPKs and p53 [204]. In other studies, Res + curcumin produced a better chemopreventive effect by maintaining adequate zinc and regulating p21 and Cox-2 level during lung carcinogenesis [207]. Genistein + Res also reduced the most severe grade of prostate cancer in the SV-40 tag rat [177].

As shown in the current study, the combination of UA + Res was a more effective inhibitor of skin tumor promotion by TPA in ICR mice than either agent alone at the

dose used and this was true for both tumor multiplicity and tumor size (see again Fig. 4-2). Although we did not design the current studies to analyze the development of squamous cell carcinomas (SCCs), papillomas are considered premalignant tumors and previous studies have shown that reductions in numbers of papillomas leads to reductions in SCCs [11, 14, 121]. As noted above, previous studies have shown that UA broadly inhibited a number of signaling pathways including EGFR, MAPK, Akt/mTOR, NF-κB, Cox-2 and STAT3 in a variety of cell types, including mouse epidermis in vivo [129, 130]. Furthermore, mechanisms associated with the anti-tumor promoting effects of Res include modulation of NF-κB, Cox-2, mTORC1, and SirT1 [106, 167, 169, 171]. In our current study, we evaluated a number of oncogenic signaling molecules including EGFR, Src, STAT3, Fas, NF-κB, Akt, p38, JNK1/2, c-Jun, and mTOR as well as the tumor suppressors p27, p21, PDCD4, and AMPK. As shown in Figs. 4-5 and 4-6 we found that the combination of UA + Res was more effective at altering these pathways during tumor promotion than either UA or Res given alone. In particular, the combination was significantly more effective at inhibiting TPA-induced activation (phosphorylation) of EGFR, Src and p38 MAPK and at altering the levels of Fas (decrease) and p-AMPK-α^{Thr172} and p21 (increase) compared to either UA or Res given alone. In addition, the combination of UA + Res produced the greatest inhibition of NF-κB, Egr-1 and AP-1 DNA binding activities and nuclear translocation (Figs. 4-9 and 4-10). All three of these transcription factors are known to be upregulated during skin tumor promotion and skin carcinogenesis [21, 208]. Thus, the combination of UA + Res produced a more global and robust inhibition of epidermal signaling pathways compared to either UA or Res given alone.

TPA-induced epidermal hyperproliferation is required for its tumor promoting activity [13, 14, 21]. Previous studies have shown that topical treatment of both 1 μmol of UA and 2 μmol of Res reduced TPA-induced BrdU incorporation in SENCAR mouse skin [107]. In the current study, we observed that the combination of UA + Res together with TPA at a dose of 2 μmol each inhibited epidermal hyperproliferation to a greater extent than with either compound alone at the same dose. The greater inhibition of TPA-induced epidermal hyperproliferation with the combination was likely due to the greater effects observed on the multiple signaling pathways noted above. Skin inflammation is also known to be an important component of the process of skin tumor promotion by TPA involving the production of pro-inflammatory cytokines and infiltration of inflammatory cells [21, 23, 209]. Again, as seen with the analyses of epidermal proliferation, the combination produced greater inhibition of inflammation. In this regard, the combination of UA + Res inhibited to a greater extent the increased expression of IL-1α, IL-1β, IL-22 and Cox-2 seen following treatment with TPA. In addition, as shown in Fig. 4-7B, the number of mast cells was decreased by either UA or Res treatment followed by TPA, however, the combination gave rise to an even greater inhibitory effect. The number of lymphocytes, monocytes and leukocytes ($CD45^+$ cells) in the dermis were also significantly inhibited by the combination of UA + Res compared to the individual compounds alone. The greater inhibition of inflammation by the combination of UA + Res was likely due to the greater inhibition of inflammatory signaling pathways and to a greater reduction in NF-κB DNA binding activity.

In conclusion, the current study shows for the first time the efficacy of a combination of UA + Res for inhibition of tumor promotion in mouse skin. This combination of UA + Res produced a greater inhibition of skin tumor promotion by TPA compared to UA or Res alone. In addition, the combination targeted multiple TPA-induced signaling pathways involved in both epidermal proliferation and inflammation and produced effects on a number of these pathways greater than either compound alone. For the current experiments we choose to apply the compounds via the topical route. An important goal for future studies will be to examine the efficacy of this and other combinations, when given in the diet. Combining phytochemicals such as UA and Res appears to produce a CR mimetic type of effect by targeting multiple signaling pathways and should be explored further for potential cancer chemopreventive efficacy.

Chapter 5. Summary, significance and future studies

Given the projections for increases in cancer incidence worldwide and cancer related morbidity and mortality as well as the high costs of cancer treatment, there remains an increasing interest and need to develop effective cancer prevention strategies. According to the World Health Organization (WHO), at least one third of all cancers can be prevented by controlling dietary factors, excessive body weight, and lack of physical activity. Numerous studies have shown that diet-induced obesity increases cancer risks and modifications of an individual's diet, such as increased consumption of fruits and vegetables, as well as increased physical activity, have been shown to be potentially effective interventions. However, it is very difficult to regulate these factors for a large portion of the population and a commitment of significant change in life style is often needed. In this regard, numerous studies have found agents that reverse, inhibit or delay the onset of early and in some cases even later stages of carcinogenesis [6]. In addition, considerable studies are being conducted to develop and/or identify agents which mimic some of the beneficial effects of CR on tumorigenesis. Therefore, the current studies focused natural compounds including triterpenes (UA, OA, AA, CA, 3-epiCA, MA, and 3-epiMA) from *P. frutescens* and Res (from a variety of natural sources) for possible cancer chemopreventive effects and CR mimetic activity on tumorigenesis. Although UA and Res have been widely studied for their anti-tumorigenic effect in many cancer models including the skin tumor model, the efficacy of the other listed triterpenenoid compounds have not yet been tested or studied in great detail in animal models of cancer.

As noted, a number of studies have discovered phytochemicals (e.g., UA, Res, curcumin, genistein) which possess cancer chemopreventive activity in many cancer

models. However, phytochemicals given as single agents may not be efficacious cancer preventive agents, especially in large populations of genetically diverse individuals. Considering they have relatively low specificity against single target proteins and low toxicity when compared with synthetic compounds, combining phytochemicals may be more advantageous for cancer prevention. In this regard, a number of studies support the hypothesis that combining of phytochemicals might be good strategy for a greater chemopreventive effectiveness [174, 175]. Therefore, the hypothesis of current study was that combining agents may provide the most rational and effective approach to cancer chemoprevention and provide overall effects that more similarly mimic the effects seen with CR. CR has been shown to inhibit tumorigenesis in many different cancer models regardless of the mode of tumor induction [210]. Therefore, in addition to evaluating a broader range of triterpenes for chemopreventive activity, this project also combined two well-established phytochemicals, UA and Res, for their possible combinatorial cancer chemopreventive effect in skin tumor promotion during two-stage skin carcinogenesis in mice.

In the current project, we conducted a comprehensive evaluation of the inhibitory effects of UA and a series of related triterpenes including OA, AA, CA, 3-epiCA, MA, and 3-epiMA from *P. frutescens* on skin tumor promotion. Several of the compounds were evaluated for the first time including, CA, 3-epiCA, MA, and 3-epiMA. Further studies explored in detail potential mechanisms for the tumor promotion inhibitory activity of this group of compounds. All of the triterpenes evaluated effectively inhibited skin tumor promotion by TPA at the dose used. Notably, several of the compounds, i.e., 3-epiCA and MA, were more effective at inhibiting skin tumor

development when compared to UA, a prototype triterpene which has been more widely studied for its cancer chemopreventive activity. All compounds also inhibited TPA-induced infiltrated inflammatory cells in dermis, inflammatory gene expression, and epidermal hyperproliferation. Again, 3-epiCA and MA were found to have the greatest inhibitory effect on tumor promotion and produced greater inhibitory effects on these responses induced by TPA that are essential for the process of tumor promotion. AMPK is activated in response to CR and increasing evidence has suggested that AMPK activators are potent inhibitors in chemically- or UV-induced skin cancer. Our data indicate that all triterpene compounds at the dose tested (excluding UA) significantly activated AMPK. These results also support the hypothesis that triterpene compounds have CR mimetic activity. Overall, the findings related to the triterpenes found in *P. frutescens* suggest that further evaluation of these compounds is warranted and that several compounds, in particular, 3-epiCA and MA would be very attractive candidates for further investigation.

The current data also indicate that combining agents such as UA (a pentacyclic triterpene) and Res (a phytoalexin) is a valid approach for achieving greater chemopreventive activity. As shown in Chapter 4, topical pretreatment with the combination of UA + Res with inhibited skin tumor promotion by TPA as shown by significant decreases in both tumor multiplicity and tumor size to a greater extent than with either compound alone. Also, the combination of UA + Res produced a greater inhibition of both cellular (epidermal hyperproliferation and skin inflammation) and biochemical/molecular (expression of inflammatory cytokines, cell signaling and transcription factor activity) changes associated with the process of skin tumor promotion than with either agent alone. Again, as mentioned above, AMPK plays an

important role in preventing skin tumorigeneiss. The combination of UA + Res induced a dramatic increase of p-AMPK-α^{Thr172}. Collectively, the data indicate that the combination of UA + Res is an effective inhibitor of skin tumor promotion which has a greater chemopreventive activity and has activity that resembles some of the effects of CR. It is also important to point out that this combination was more effective in mice maintained on either a 10 Kcal % fat (overweight control diet) or a 60 Kcal % fat diet (obesity-inducing diet) producing mice with overweight and obese body phenotypes, respectively. The data indicate combinations such as the one used in our current studies may be useful in an obese setting where risk reduction is needed but where rapid weight loss cannot be effectively achieved.

With constant efforts to elucidate mechanisms of CR in tumorigenesis and to identify target proteins for cancer chemoprevention studies, various mechanisms have been discovered that are attributed to cancer chemopreventive agents. It is clear that multiple signaling pathways are known to be involved in the effects of CR during cancer development. Furthermore, obesity increases cancer risks by inducing inflammatory responses leading to tumor development. Thus, targeting multiple signaling pathways and inflammatory responses simultaneously appears to be the most rational approach for achieving chemopreventive efficacy. Since the triterpene compounds tested in this study displayed inhibitory effects against multiple signaling pathways, skin inflammation, and proinflammatoy cytokines in response to TPA, we were able to conclude that the triterpenes found in *P. frutescens* possess good chemopreventive activity and CR mimetic activity. Although all of the compounds triterpenes studied had some overlapping activity, each of the compounds also possessed some differential specificity towards particular signaling pathways. For

example, our data indicate that while UA did not further activate AMPK, the other triterpenes examined did further activate AMPK when given with TPA. Furthermore, several of the compounds, notably 3-epiCA and MA, inhibited some signaling pathways (i.e., IGF-1Rβ) not significantly inhibited by the other triterpenes. The structural features that lead to these differences in activity among the triterpenes are not known at the present time but would seem an interesting area for further research. In addition, although UA did not activate AMPK when given by itself with TPA, Res significantly increased AMPK activation. However, the combination of UA + Res exhibited a dramatic activation of AMPK. Hence, combining agents may lead to potentiated effects not only on signaling pathways but also on inhibition of carcinogenesis that warrants further evaluation.

Overall, the present data support the hypothesis that triterpenes alone or in combination with other chemicals such as Res act like a CR mimetic by modulating growth factor signaling, inflammatory signaling, skin inflammation, and inflammatory cytokines in relation to inhibitory effects on skin tumor development. Further study of CR mimetic combinations could lead to identification of combinations that would be suitable for use in human populations especially high risk populations as a cancer chemoprevention strategy.

Ongoing and future studies

Future experiments will explore the efficacy of 3-epiCA and MA that appeared to possess the greatest anti-tumor promotion effects in this project. For future studies, these compounds will be given in a diet at various dosages (e.g., 0, 1.0, 10.0 g/kg) and using two different diet regimens (control and obese diets) and evaluated for

their ability to inhibit skin tumor promotion by TPA. In this study, we will be able to see the efficacy of dietary administration of the compounds on skin tumor promotion. The dietary efficacy of these compounds will also be compared with that of UA. Bioavailability of these compounds will be determined as assessed by serum analysis of the parent compounds and treatment with several doses of UA, 3-epiCA and MA will allow us to establish a dose-response relationship. In addition, comparisons of their efficacy between control and obese mice will provide better evaluation of their effects in obese mice on skin tumor formation. Data generated as part of our completed study demonstrated that UA and related compounds differentially regulated multiple signaling pathways. STAT3 signaling is one of the most affected pathways by several compounds including CA, 3-epiCA, MA, and 3-epiMA. Therefore downstream molecules such as c-myc, Bcl-xL, survivin, bcl-2, and cyclin D1 in addition to Twist1 will be further evaluated. We also observed significant activation in AMPK and its direct downstream target, Ulk1 ^{S555}, which is responsible for activation of the autophagic pathway, by pretreatment with AA, CA, 3-epiCA, MA, and 3-epiMA. Therefore, further evaluation of this pathway in the chemopreventive effects of these compounds will be conducted by analyzing autophagic molecules including Atg5, beclin1, and LC3IIB. In addition to Western blot analysis, electron microscopy and immunofluorescence assays will allow for better evaluation of compound efficacy on autophagosome formation.

In ongoing experiments, the ability of UA in combination with another widely-studied chemopreventive agent, curcumin, is being evaluated for possible inhibition of skin tumor promoting. The combination of UA + curcumin (various doses) is being applied topically 30 prior to TPA treatment to see whether the combination has additive or

synergistic effects on inhibiting TPA-induced epidermal signaling pathways including Akt/mTORC1, p38 MAPK, JNK1/2, NF-κB, and AMPK. In addition, the effect of UA + curcumin will be evaluated on TPA-induced epidermal hyperproliferation and skin inflammation. Lastly, the ability of UA + curcumin to act additively or synergistically at inhibiting skin tumor promotion will be examined. Preliminary data suggest that this combination also has greater ability than either agent alone to inhibit epidermal proliferation and signaling induced by TPA.

In another ongoing experiment, the dietary effect of UA (2 g/kg of AIN76A diet) in combination with Res (5g/kg of AIN76A diet) or curcumin (10g/kg of ANI76A diet) on short-term markers of skin tumor promotion is being evaluated. Bioavailability of these combinations will be determined based on measurement of their amount in sera. The multiple signaling pathways identified to be affected by the combination of topical UA + Res will be also explored for this study. The effect of UA in combination with Res or curcumin on TPA-induced epidermal thickness, LI, and expression of inflammatory cytokines will be investigated. Thus, results from short-term experiments will provide comprehensive mechanisms in relation to anti-tumor promoting effect. Finally, two-stage carcinogenesis experiment will be conducted to evaluate the effect of oral administration with UA + Res or UA + curcumin on skin tumor promotion by TPA. Hence, our ongoing and further experiments will allow us to identify potential chemopreventive agent combinations administered in the diet and the results obtained from these experiments may allow further assessment as to the potential usefulness of such combinations for human intervention studies.

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